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Cripto-1 (Cr-1) and Amphiregulin (Ar) are two EGF-like growth factors that occur at higher levels than normal in breast cancer cells and tissues. They occur during mammary cell proliferation in normal virgin, pregnant and lactating *mouse* mammary glands, and in early embryo cells. To test the roles of Cr-1, we infected normal mouse mammary cells with retroviruses over-expressing Cr-1, or an antisense version, in order to inhibit the production of this polypeptide. Cr-1 stimulated proliferation and was inhibitory to differentiation (lactation). Reduction of Cr-1 did not affect differentiation but decreased growth rate and increased apoptosis. Cr-1 overexpression transforms cells but appears not to be tumorigenic. We completely inactivated the Cr-1 genes in order to produce mice that expressed no Cr-1 in mammary glands as well as in all other tissues. To this end, we inactivated Cr-1 in embryo stem (ES) cells by homologous recombination and produced Cr-1 gene-targeted mice. Studies on these Cr-1(-/-) cells showed that ES cell differentiation to cardiac myocytes was prevented. The "knockout" animals demonstrate that Cr-1 is essential for axis formation and embryonic development. Cr-1 absence causes aberrant proliferation of mesoderm cells, loss of migratory signals and absence of heart development.

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E. J. Adamson 8/18/98

PI - Signature Date

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(5) INTRODUCTION

This research project focuses on the expression and role of the growth factors Cripto-1 (Cr-1) and Amphiregulin (Ar) in mouse mammary gland development and cancer.

The expression of the EGF-like ligands, Ar and Cr-1 gene products, as revealed by immunocytochemistry, was thought to be restricted to tumors of the breast, ovary, colon and a few other tissues, with little or no expression in adjacent normal tissues. However, there are some situations where Ar and Cr-1 expression do occur normally, and their activities and roles are of interest because we may start to understand the conditions that deregulate their expression or their activities. Therefore we have emphasized this aspect of the molecular biology of Ar and Cr-1 to an increased degree, not in the original design.

This change was necessitated because a large portion of the described project was taken and completed by the first post-doctoral worker hired for this project, in the next post he obtained, after he was asked to leave here. The projects that have been completed elsewhere by Dr. Kenney comprise two papers, one on Ar overexpression in mouse mammary gland (3), and one on Ar and Cr-1 expression in mouse mammary tumors in transgenic mice (2). The original aims of this project were therefore completed by Dr. Kenney's contributions, but not in this laboratory. We realized that we needed to change our emphasis so as to avoid repeating the same work.

The epidermal growth factor (EGF) receptor is known to bind and respond to Ar, but Ar has a distinctive effect compared to the other 5 ligands for cells that respond to its signal. Ar is known to be a product of many human breast cancer cell lines, but whether it has a role in the tumorigenic process is one question that we addressed. In contrast, Cr-1 is an orphan ligand, with no known receptor, since the truncation of the sequence within the EGF-like domain makes this ligand unable to bind to the EGF receptor. New data from the laboratory of Dr. M. Kirschner, indicates that Cripto-like ligands produced in Xenopus laevis (frog) may be related to the fibroblast growth factor (FGF) family of ligands and may bind to an FGF receptor molecule but this has not yet been established (4). New data from the laboratory of Dr. P. Leder, indicated that an additional Cr-1-related molecule, *Cryptic*, is produced in mouse. Cryptic has a different expression range than Cr-1, but is also expressed in the postimplantation embryo. Thus Cr-1 is a now one member of a family of genes with the proposed name CFC(5). A new member of the family has been identified in the Zebrafish, as the *one-eyed pinhead (oep),* a gene product essential for gastrulation (6).

The main theme of our original research project on the mammary gland was based on findings that gene products that have EGF-like sequence domains can act by a) stimulating autocrine or paracrine growth, and b) stimulating the synthesis of some of the other members of the ligand family and c) inducing the prolonged expression of the epidermal growth factor receptor (EGFR). These events are hypothesized to lead to stimulation of growth and to conditions that are conducive to genetic changes that may lead to cancer. The question that we were addressing was whether Ar and Cr-1 can **initiate** the tumorigenic process in mammary gland. This was to be done by over- and under-expression of the growth factors in mammary cells in vitro and reimplantation of the cells into the mammary gland to test their new potential for tumor formation or, in the case of under-expression, for inhibition of the development of the mammary gland. The interaction between the stroma and the epithelial cells was to be examined, to determine if there was a differential role in the event of tumor formation.

The new emphasis on the developmental aspects of Cr-1 and Ar led us eventually to an updated goal of determining what the result would be of the "knockout" of these genes in the mouse. We were offered the exclusive use of a Cr-1 targeting vector by Dr. M. G. Persico, and one for targeting Ar, constructed by Dr. G. Plowman. We collaborated with Dr. L. M. Wiley, to determine if Ar played a role in early development. These studies were done in parallel with the studies on Cr-1 roles in mammary gland, and we have also pursued the question of the roles of Cr-1 and Ar in mammary tumor formation in transgenic mice, and report that Cr-1 could be useful as an early marker of mammary cell hyperplasia.

The initial SOW was modified in order to respond to the events ensuing from changes in personnel, and to tackle the concept of gene inhibition with a more robust plan. The changes of plan were described in previous years reports. Other changes in our detailed plans occurred in response to our own findings and the results of others. The overall aims are important and remain the same.

(6) BODY

I. The expression and roles of Ar and Cr-1 in mouse mammary epithelial cells.

1. We have shown that Ar and Cr-1 are produced during the normal postnatal development of the mammary gland in mouse (1) *(publication 1).* These growth factors are just perceptible among the proteins in the 12 week old mammary gland (using immunoblotting, immunocytochemistry and reverse transcription polymerase chain reaction, RT-PCR), but greatly increase during pregnancy and are at the highest level in late pregnant and early lactating glands. (Summary only - work reported earlier)

2. We have completed the study of the roles of Cr-1 in mammary glands by over-expressing and by inhibiting Cr-1 expression in a normal mouse mammary cell line, CID 9, *(publication 2*, a copy is in the Appendix). The following is a summary.

Project to over- and under-express Cr in mammary cells.

a) *Methods.* The principal method to reveal the biological role of a gene product is to manipulate its expression in cells or in the whole animal. We can report significant progress in both these approaches. Dr. Christine Niemeyer constructed a retroviral expression vector that can be used to infect mouse cells (CID 9 mammary cells) so that they over-express Cr-1 in one population, or have reduced Cr-1 expression in another. The control population was infected with an "empty" retrovirus. This is a necessary control to indicate if the infection produced an effect independently of the gene insert.

b) The *results* reported last year were extended, and are summarized as follows. Cripto was found to be expressed in CID 9 cells, a line of mammary epithelial cells derived from 14.5 day pregnant mice and we have used these cells to investigate the roles of this gene. Our results showed that aberrant expression of Cripto affected the growth, morphology, and differentiation of these cells. Exogenous mouse Cripto expression from a retroviral vector caused CID 9 cells to grow at an increased rate and to increased cell densities compared to parental and control cells. CID 9 cells overexpressing Cripto grew anchorage-independently and did not differentiate efficiently. Infection of CID 9 cells with a Cripto antisense vector caused these cells to change in morphology, to grow slowly and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cripto is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of the cell interactions that precede differentiation. The net effect is the stimulation of proliferation at the expense of breast cell differentiation by Cripto. In addition we showed that cells with reduced Cr-1 expression were more likely to undergo apoptosis, and hence it is likely required for development and growth.

When these Cr-1-under and over-expressing CID-9 cells were transplanted into mammary fat pads of syngeneic mice, they all produced mammary tumors, although, the literature indicated that the cells were, at one point, *not* tumorigenic. The disadvantage of using cell lines is that they change with increasing passage number. We were aware of this possibility and started with low passage cells specially obtained from Dr. Mina Bissell. We next obtained a similar mammary cell line, COMMA-D that is less tumorigenic and gave no tumors after transplantation but did produce hyperplasia after 10 weeks, in 15 out of 18 cases, whether the cells were wild-type controls or over-expressing Cr-1. Interestingly, COMMA-D cells expressing little or no Cr-1 due to antisense expression, did not grow at all, either normally or abnormally. One new conclusion was possible based on the results in these cells: the removal of expression of Cr-1 in COMMA-D mammary epithelial cells counteracts their inherent growth capacity after transplantation. Furthermore, we also showed that the removal of Cr-1 from mammary cells increases the normally low level of apoptosis three-fold. This increase in programmed

cell death explains why mammary cells lacking Cr-1 did not survive after transplantation. This result confirmed the previous finding that Cr-1 is necessary for normal mammary gland growth and development.

In summary, Cr-1 is required for normal mammary cell survival, growth and for epithelium development, but probably does not transform mammary cells. Although not proven by our results, the data suggest that Cr-1 is not an initiator of the tumorigenic process.

3. Project to over-express Ar in mammary epithelium.

a) *Methods*. The experimental approaches originally described, are in progress also for Ar, but modified by using COMMA-D or the related HC11 normal mammary epithelial cells, and are being performed by Dr. Bradley Spencer-Dene. We have prepared Ar expressing retroviruses and plasmid vectors to choose the best expression vector. Dr. Spencer-Dene prepared a plasmid expression vector that over-expresses Ar using an inducible promoter, MMTV, which is induced by the glucocorticoid analog dexamethasone, as well as lactogenic hormones. The additional feature that is included is an epitope to a tag "FLAG", that can theoretically be detected using a commercial anti-FLAG antibody. This allows us to assay the expression of the exogenous vector in cells that also express endogenous Ar. However, it turns out that the epitope is not detectable in cells transfected with the vector and, therefore, must be masked during folding of the protein. The sense version of the vector is able to stimulate the expression of Ar in HC11 mouse mammary cells. This induction can be induced by 1 h treatment with 1 μM dexamethasone. We decided that making a transgenic mouse was the best approach to study the over-expression of Ar in the mammary gland. We therefore prepared the vector for injection into mouse eggs to make transgenic mice that would express Ar almost exclusively in the mammary, prostate, epididymis and salivary glands of transgenic mice. This is described next.

4. Project to make MMTV-Ar transgenic mice.

a) *Methods.* We have decided that the most urgent and unique study not so far performed or described in the literature is the over-expression of Ar in transgenic animals. The procedures are performed by the transgenic mouse facility at the Institute. We have made a construct to overexpress Ar specifically in mammary glands of TGM, using MMTV-Ar expression vector. We tested its activity in mouse mammary cells, and in NIH3T3 cells with dexamethasone to stimulate the estrogen response. The vector proved to be expressed and was inducible. A range of Ar sizes was produced (50 kDa down to 14.5 kDa), all have been observed in other cell types under these conditions. With this encouragement, we injected the linearized construct into about 100 mouse eggs and eventually 4 live transgenic founders were detected.

b) *Results.* We have bred each line twice and await further offspring before sacrificing the females to determine that the Ar transgene is being expressed in the mammary glands. We predict that after 2-3 pregnancies, Ar TGM will produce mammary tumors. We await further results on this question.

5. Project to make antibodies to Cr-1 and Ar.

We have now successfully raised rabbit antibodies to Ar. a) *Methods.* We purified the original 17-mer peptide by HPLC and have made specially formulated immunogen as a mixture of 2 types of crosslinking with carrier protein, in order to increase the immunogenicity of Ar. We need the antibody to assay the level of expression of the Ar protein product in our studies to over- and under-express Ar in mammary cells. The results in Figure 2 were obtained with the new antibody. The bacterially synthesized Cr-1 that we made proved to be highly insoluble (aggregation due to the high Cysteine content), and little useful Cr-1 could be prepared.

b) *Note.* We are continuing to use the antiserum raised by Dr. Persico to a peptide within the mouse Cr-1 sequence. This gives a large number of high Mol. Wt bands on gels that we cannot account for, so this remains a significant problem. Expression studies can be performed by assaying the level of mRNA, but will not answer the question of whether the mRNA is translated and whether the role of the gene product is really being tested. We are attempting to obtain antibody from another source, but this has not yet succeeded.

6. Project to determine the time course of the expression of Cr-1 and Ar during tumorigenic progression.

a) *Methods.* Transgenic mice were provided by Dr. W. Muller, or were purchased from the Jackson lab. We have examined the mammary glands before and during the tumorigenic event in several transgenic mouse (TGM) models using Western blotting. One TGM line was made by the insertion of the Polyoma middle T antigen behind the mouse mammary tumor virus (MMTV) LTR originated by Wm Muller. We have also obtained TGM from the Jackson lab that overexpress MMTV-c-neu (ErbB2), and TGM that overexpress mMT-TGFα in mammary glands. The c-neu-expressing mice give rise to tumors after about 30 weeks of age, but for the TGFα TGM, tumors only arise after 12 months of age.

b) *Results.* i) The females of PyT TGM can develop multifocal mammary tumors starting at around 34 days of age with hyperplasia and progressing rapidly to large adenocarcinomas. Tumorigenesis in the males takes longer. We found that both Ar and Cr-1 are over-expressed in the large late tumors, and we have now completed a time course to determine that both Ar and Cr-1 are over-expressed at the hyperplasia stage before frank tumors are seen. EGFR and ErbB2 not overexpressed at this hyperplastic stage but ErbB2 increases in tumors.

ii) The MMTV-c-neu mammary glands produced Ar over-expression only in frank tumors and not in hyperplastic tissue. They expressed Cr-1 early in the hyperplastic tissue and this expression unexpectedly dropped in frank tumors. Both EGFR and ErbB2 expression was highly elevated in tumors.

iii) The MT-TGFα TGM produced a variety of Ar forms, but again hyperplasia showed elevated Ar and Cr-1 whereas the adenocarcinomas were reduced in expression. Only the pregnant mammary gland produced as much Cr-1 as the hyperplastic mammary gland, thus definitely linking elevated Cr-1 expression in hyperplasia in 3 of 3 TGM mammary tumors. Ar was expressed in hyperplasia in 2 of 3 TGM glands. This result has been submitted for publication *(publication 3).*

II. Studies on the developmental roles of Cripto-1.

1. Progress report on the inactivation of Cr-1 in ES cells and in vivo.

Methods. Dr. Chunhui Xu in the laboratory with DNA provided by Dr. Graziella Persico, Naples Italy, has made the second targeting vector for homologous recombination and targeting, that recognizes the mouse Cr-1 gene. She has used the vector to perform the technique of homologous recombination in order to inactivate both alleles of the Cr-1 gene in ES cells. Standard procedures were used to perform the knockout in ES cells and these were all done by Dr. Xu. The microinjection of the resulting ES cells into blastocysts was performed by the transgenic mouse facility personnel. There are 2 parts to this study:-

a) Two of the ES cell clones that were assayed and found to contain a single inactivated Cr-1 gene, were microinjected into mouse blastocysts (a pay back service at the Burnham Institute) following published procedures for making "knockout " mice. We had 20 male chimeras that proved to have germ-line abrogation of the Cr-1 gene and hence produced progeny that were heterozygous Cr-1 knockout mice. We bred these mice and produced Cr-1 (-/-) mice without any Cr-1 expression.

i) *Results.* The homozygous embryos died at gestation day E7 to E9, due to the inability of the mesoderm and ectoderm to properly undergo morphogenesis. As a result there is no recognizable development after the initial formation of the neuroepithelium, there is no further differentiation of the cardiac mesoderm and no beating heart. The visceral yolk sac alone continued to develop up to E10 but there were no embryos within the yolk sacs. We tested the expression of the following developmentally important marker genes: Brachury (for early mesoderm -positive); Alphafetoprotein (for visceral endoderm -positive); Laminin (for parietal yolk sac and later all epithelial cells - positive); Flk-1, flt-1, tie-1 and tie-1 (markers of endothelial cells (positive); transcription factors GATA-4, NKX2.5, MEF2C

(all positive); atrionaturietic factor (negative); cardiac myosin light chains MLC2v, MLC2a, cardiac myosin heavy chain and cardiac actin (all negative). We concluded that Cr-1 is necessary for correct developmental morphology and more specifically for the development and differentiation of cardiac muscle (paper submitted for publication, *publication 4,* preprint attached).

b) The inactivation of the second Cr-1 allele in ES cells has produced cells with a major defect that is only obvious when the cells are cultured under conditions that lead to their differentiation. One of the most frequent types of cell produced by differentiated ES cells is the beating cardiac myocyte which are seen on the 7th day and beyond. The ES-Cr(-/-) cells were unable to differentiate into cardiac myocytes. No beating was seen in 21 days of culture and no cardiac myosin protein was detected in immunoblots. This was specific because alphafetoprotein (AFP) was produced in normal amounts in all ES cell types, Cr-1(+/+), (+/-) and (-/-). AFP is another product secreted from differentiated ES cells. Moreover, the effect was dose dependent, because the (+/-) ES cells were 2 days delayed in their ability to give beating heart muscle. In addition, we "rescued" the mutant behavior by transfecting the Cr-1 null ES cells with an expression vector for Cr-1. In those cells only and not the antisense transfected cells, cardiac differentiation was restored. We tested for all the early markers of differentiation to the cardiac lineage and found that Cr-1 null cells are unable to switch on any of the cardiac myosin genes. They do express all of the transcription factors that are known to be in the cardiac pathway, and so we are very excited that Cr-1 appears to be a major switch gene for the differentiation of cardiac muscle. Importantly, skeletal muscle was not affected, and Cr-1 null cells produced striated muscle myotubes in culture. We have published these results *(publication 5).*

c) Further recent studies on our cell lines that lack Cr-1 indicate that Cr-1 stimulates the growth of embryo cells, is a chemotactic agent as well as needed for migration of cells. These findings explain why mammary carcinoma cells (producing high levels of Cr-1) are more highly proliferative and are more mobile. This could stimulate metastases of mammary tumor cells in patients (paper in preparation).

2. Rescue of the lethality due to Cr-1 abrogation using chimeric animal constructs.

a) *Methods*. We used our ES(KO)2 cells to make chimeric animals by their microinjection into normal C57Bl6 blastocyst embryos. The idea is that, if null cells are acting autonomously and are unable to take part in the development of a specific tissue, such as the heart, the resulting chimeric animals will have only normal cells in the heart and Cr-1-null cells will be excluded.

b) *Results.* The result was that the Cr-1-null ES cells took part in the development of every tissue in the resulting adult animals, and these were alive and well. Our conclusion was that since Cr-1 is produced as a soluble protein, it was able to rescue the null phenotype of the null component of the mice so that it was able to take part in development. We proved by this result that Cr-1 is a soluble, diffusible protein with a range of distal activities that compensate for inactive Cr-1 genes *(paper 4,* submitted for publication).

III. Studies on the developmental role of Amphiregulin.

1. Collaborative project with L.M. Wiley of UC, Davis. Dr. Wiley's expertise is preimplantation development. My laboratory found that embryo stem (ES) cells, that are derived from blastocyst embryos, express Ar. This meant that Ar could be expressed in preimplantation embryos. We provided the antibody that would detect Ar by immunocytochemistry, and the PCR oligonucleotides to Dr. Wiley. Dr. Tsark in Dr. Wiley's lab showed that Ar is produced as early as the 8-cell stage of embryogenesis and is an autocrine factor that increases both rate of development /differentiation (time of onset of cavitation and trophoblast formation) and cell proliferation. It is significant that Ar influences the processes of growth and differentiation even in early embryo cells. It indicates the possibility that the stimulation of growth by Ar occurs not just in adult cells but also in cells that are immature or dedifferentiated, a state that describes tumor cells *(publication 6).*

2. The knockout of the Ar gene in mouse failed. We deemed the reason was that there was a fault in the

targeting vector. The laboratory of Dr. Robert Coffey (Vanderbilt) was also unable to obtain any knockout ES clones. This project was abandoned.

(7) CONCLUSIONS

Cripto-1.

1. Cr-1 protein contributes to the growth and development of normal mouse mammary epithelial cells. Cr-1 production is up-regulated by pregnancy hormones, while regressing mammary glands produce no Cr-1.
The data indicate that Cr-1 plays a role in the events leading to differentiation (lactation) but **not** in differentiation itself. Reduction of Cr-1 levels inhibits growth in monolayers and in soft agar, but does not affect their ability to differentiate as shown by the production of milk proteins.

2. Cr-1 reduction increases apoptosis. Cr-1 is a survival factor for mammary epithelial cells.

3. Data from homologous recombination studies (gene targeting) in ES and EC cells indicates that Cr-1 is needed to maintain the high rate of proliferation of undifferentiated EC cells.

4. Cr-1 plays a role in the events leading to cardiac cell differentiation at a stage before the synthesis of myosin. The loss of Cr-1 does not affect the ability of differentiating ES cells to produce a large number of other markers of differentiation.

5. Cr-1 is necessary for embryonic development from the E7 stage, and absence leads to lethality soon after. A combination of defects in cell proliferation and cell migration in the absence of Cr-1 explains embryonic lethality.

6. Cr-1 is a chemotactic and migratory factor to cells that have the Cr-1 receptor (not yet identified) and this is relevant to mammary tumor cells which secrete high levels and to metastatic behavior.

7. Cr-1 is probably *not* an initiating transforming growth factor for mammary cells.

8. Cr-1 is a marker of the hyperplastic stage of the tumorigenic process.

Amphiregulin.

1. Ar is expressed in early mouse embryos as early as the 8-cell stage, in the inner cell mass and the trophoblast cells of the blastocyst. It plays a part in the development of the preimplantation embryo based on inhibition of cell numbers in embryos incubated in vitro with antisense Ar oligonucleotides.

2. Ar is expressed in the late stages of postnatal mammary gland development. It is produced maximally in pregnant glands and is less prominent in lactating glands. Ar is an autocrine growth factor for mammary cell proliferation.

3. Ar production is increased in mammary glands from MMTV-PymidT mice before the production of overt tumors. More evidence is needed to order to identify this growth factor as a contributor to the tumorigenic process, or as a marker or precancerous conditions.

Completed tasks for Cr-1

We have completed tasks 1-3 of the original SOW (omitting TGFα as explained earlier).
We have not attempted to do the time points indicated in task 4 because the cells were already rapidly tumorigenic, even without further expression of Cr-1.
We have completed task 5 for the current CID9 cells and COMMA-D cells, which are less tumorigenic.
We have totally abrogated the activity of Cripto genes in ES and F9 EC cells and analyzed the effects.
We now have Cr-1(+/-) mice which are viable and healthy. We bred these mice to produce homozygous

Cr-1(-/-) embryos that die on gestation day 7 to 8. Therefore Cr-1 is important in early postimplantation development.

Completed tasks for Ar

We have succeeded in making a rabbit polyclonal antibody to Ar, that works in immunoblotting (Westerns).

We have completed the construction and testing of a mammary specific expression vector (MMTV-LTR-Ar).

We have injected the construct into mouse eggs and have 4 founder mice that have inserted the vector sequences into the germ-line. We are still testing whether these mice over-express Ar in mammary glands.

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PERSONNEL receiving pay from this project

Eileen D. Adamson, 1994-8, PI.
Nicholas Kenney, 1994-5, PDF.
Christina Niemeyer, 1994-7, PDF.
Bradley Spencer-Dene, 1995-8, PDF.
Chunhui Xu, 1996-7, PDF.
Gerhart Graupner, 1995, Post-doctoral consultant.
Weiping Tan, 1996-8, technician.

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Cripto: roles in mammary cell growth, survival, differentiation and transformation

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Abstract

Cripto-1 (Cr-1) protein, encoded by the teratocarcinoma-derived growth factor gene (*TDGF-1*), is highly correlated with transformation in breast cancer. Eighty-two percent of breast carcinomas express Cr-1 whereas it is undetected in normal human breast tissue. We confirmed and extended findings that Cr-1 protein is expressed during the pregnancy and lactating stages of normal murine mammary glands but is barely detectable in glands from virgin animals and is undetectable in involuted glands. Cr-1 was found to be expressed in CID 9 cells, a line of mammary epithelial cells derived from 14.5 day pregnant mice and we have used these cells to investigate the roles of this gene. Exogenous mouse Cr-1 expression from a retroviral vector caused CID 9 cells to grow at an increased rate and to increased cell densities compared to parental and control cells. CID 9 cells over-expressing Cr-1 did not differentiate efficiently. Infection of CID 9 cells with a Cr-1 antisense vector caused these cells to change in morphology, to grow slowly, to undergo apoptosis at a higher rate and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cr-1 is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of differentiation. In transplantation studies, Cr-1 over-expression stimulated the growth and survival of mammary cells, but did not stimulate tumorigenesis *in vivo*.

Keywords: retroviruses; overexpression; antisense; milk proteins; apoptosis; anchorage independent growth; tumorigenicity

Abbreviations: Cr-1 or CR-1, Cripto; DMEM, Dulbeccos minimum essential medium; EGF, epidermal growth factor; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbant assay; FBS, fetal bovine serum; IRES, internal ribosome entry site, PBS, phosphate-buffered saline; TDGF-1, teratocarcinoma derived growth factor-1 gene

Introduction

Cripto (Cr-1) was first assigned to the Epidermal Growth Factor (EGF)-like family of ligands that includes transforming growth factor-alpha (TGFα), and amphiregulin (AR). This family of proteins contain 'EGF-like domains' with a highly conserved structure of three disulfide loops, and in Cr-1, the six conserved cysteines that make up the EGF motif lacks the A-loop and the B-loop is truncated. As a result, Cr-1 does not bind to the EGFR or members of the ErbB family of receptors. At the carboxyterminus of Cr-1, an additional six-cysteine motif is present that is conserved in an analogous protein recently isolated from *Xenopus laevis* (Kinoshita *et al*, 1995). A related mouse protein, Cryptic, was recently cloned and this group of genes forms a new family named CFC (Shen *et al*, 1997). Mouse Cr-1 protein consists of 171 amino acids and unlike the human CR-1 (188 amino acids) has a signal sequence and is secreted (Brandt *et al*, 1994) (Dono *et al*, 1993) (Normanno *et al*, 1994) (Ciccodicola *et al*, 1989).

CR-1 was cloned as a full length isolate from a cDNA library derived from the human teratocarcinoma cell line NTERA2 clone D1. The TDGF-1 (CR-1) gene and an intronless sequence CR-3 were isolated and mapped on human chromosomes 3 and X, respectively (Dono *et al*, 1991; Saccone *et al*, 1995). Mouse *Tdgf1* (encoding Cr-1), and two intronless pseudogenes, *Tdgf2* and *Tdgf3*, have been isolated and characterized (Dono *et al*, 1993; Liguori *et al*, 1996). Mouse Cr-1 has 93% similarity to its human counterpart in the EGF-like domain, which is the most conserved. The molecular mass of Cripto protein varies according to the species and cell type: in human GEO colon and NTERA2/D1 embryonal carcinoma cells a polypeptide of 36 kDa predominates and it can be differently processed by glycosylation or modified in other ways. In mouse F9 cells only the secreted protein has been analyzed and a single species at 24 kDa was noted (Brandt *et al*, 1994).

CR-1 protein is highly correlated with tumorigenicity. Of 68 biopsies on breast carcinomas, 82% expressed Cripto. Cripto expression has not been detected in normal human breast tissue or cell lines (Qi *et al*, 1994). Growth of two human breast carcinoma cell lines and the nontransformed human epithelial cell line 184A1N4 was stimulated by the addition of synthetic refolded human CR-1 peptides containing the EGF-like domain (Brandt *et al*, 1994). All the human mammary tumor cell lines examined were found to express CR-1 using RT–PCR, Northern blot analysis and immunocytochemistry (Normanno *et al*, 1994). The human CR-1 cDNA has been overexpressed in mouse NIH3T3 cells and was shown to transform these cells such that they grew in soft agar in contrast to control cells

(Ciccodicola *et al*, 1989). The same construct transformed an immortal mouse mammary cell line, NOG-8 (Ciccodicola *et al*, 1989). Mammary tumors formed in transgenic mice overexpressing oncogenes TGFα, neu, int-3, polyomavirus middle T antigen or simian virus 40 large T antigen, all express Cripto-1 (Kenney *et al*, 1995). In addition, Cr-1 expression was observed in pregnant and lactating mouse mammary glands (Kenney *et al*, 1995). The latter observation suggested that Cripto played a role in differentiation as well as proliferation.

In the present study, CID 9 cells were used to examine the expression of Cripto-1 and to determine the effects of over- and under-expression of this growth factor during growth *versus* differentiation. CID 9 cells are a subpopulation (Schmidhauser *et al*, 1990) of the COMMA-1D mammary epithelial cell line which was established from normal 14.5 day pregnant Balb/c mouse mammary gland tissue. They retain important characteristics of normal morphogenesis and functional differentiation *in vitro* (Danielson *et al*, 1984). Normal CID 9 cells differentiate into alveolar-like structures (mammospheres) that express β-casein when grown on a laminin-rich extracellular matrix in the absence of fetal bovine serum (FBS) and in the presence of lactogenic hormones (Schmidhauser *et al*, 1990).

We show here that Cr-1 is differentially expressed during mammary gland development and is expressed in a hormone dependent fashion in the normal mammary epithelial cell line. Inhibition of Cr-1 expression caused a change in cell morphology, decreased cell growth, increased programmed cell death and reduced anchorage independent growth. Mouse Cr-1 overexpression stimulated anchorage dependent and independent cell growth and decreased the differentiation potential of the mammary cells. However, no increases in the tumorigenicity of Cr-1 over-expressing mammary cells were noted after transplantation into syngeneic hosts.

Results

Cripto is a pregnancy and lactation stage specific protein

Cripto protein, Mr 24 and 26 kDa, is strongly expressed in the second phase of mammary gland development, pregnancy (Figure 1, lane 2 shows Cr-1 in 14.5 day pregnant glands). This implies that its expression is driven by pregnancy-associated hormones, since involution of mammary gland tissue after pregnancy is associated with loss of Cripto expression (Figure 1, lane 4). In contrast, the virgin mouse mammary gland expresses extremely low levels (Figure 1, lane 1) in partial agreement with Kenney *et al.* (1995).

Cr-1 is also expressed in CID 9 cells, derived from 14.5 day pregnant mammary glands, as a 24 kDa protein (Figure 1, lane 7). When the cells were cultured on basement membrane substrates, they differentiated, observed as mammosphere and expression of casein, and they also expressed higher levels of Cr-1 protein. The Cr-1 protein was also modified to Mr 26 and 28 kDa forms (Figure 1, lane 6). The CID 9 mammosphere structures appeared to be functionally similar to the *in vivo* pregnant-lactating mammary gland with stage-specific Cripto gene expression. This observation prompted us to test the role of Cr-1 by experimental manipulation of C-1 expression in CID 9 cells.

Expression of exogenous Cripto

In order to affect the expression of Cripto in mammary cells, a retroviral vector containing either sense or antisense Cripto was constructed. The polycistronic retroviral vector pGCEN (Figure 2) contains the encephalomyocarditis virus internal ribosome entry site (IRES) which allows efficient expression of multiple genes from a single proviral genome. Transcriptional controls and RNA processing steps that differentially affect expression of the exogenous genes can be avoided (Ghattas *et al*, 1991). Cripto cDNA sequences (both sense and antisense) were inserted as described in the Materials and Methods section. Cripto cDNA and the selectable marker, neo^r are both expressed from a single promoter using the EMCV IRES insert. The pGCEN vector LTR is a promoter known to function in the mammary gland (Bradbury *et al*, 1991). CID 9 cells were infected with the retroviruses. Because the CID 9 cells are a heterogeneous cell

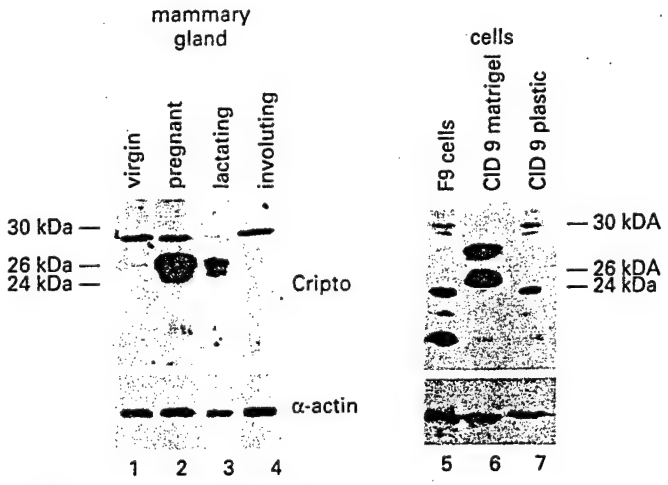

Figure 1 Immunoblots of normal mammary gland tissue and cells to show Cripto protein expression. Cripto is highly expressed as 24 and 26 kDa proteins in 14.5 day pregnant gland (lane 2) and at a lower level at the lactating stage (lane 3) of mammary gland development. It is expressed at very low levels in virgin glands (lane 1) and is not seen in involuting glands (lane 4). Cripto is expressed as a 24 kDa protein in undifferentiated F9 cells (lane 5) and as both 26 and 28 kDa proteins in CID 9 cells grown on an extracellular matrix (Matrigel) for 7 days in the presence of lactogenic hormones (lane 6), compared to CID 9 cells grown in maintenance media on plastic (lane 7). Other bands in the figure are non-specific bands

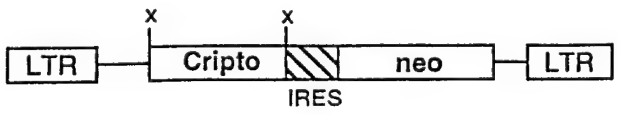

Figure 2 pGCEN retroviral expression vector used to over and under express Cripto in mammary cells. The Cripto cDNA was inserted in both orientations at the *Xho*I site (X). IRES, encephalomyocarditis virus internal ribosome entry site; LTR, Moloney murine leukemia virus Long Terminal Repeat sequences; neo, bacterial neomycin resistance gene

population, more than 250 clones were selected in G418 and then pooled.

The level of Cripto protein expression in the infected cells was measured using Western blot analysis. Two different populations of CID 9 cells containing Cripto in the sense orientation were analyzed (Figure 3) and showed that Cripto was overexpressed at levels greater than twofold higher than control vector populations (Figure 3). In the antisense populations only approximately one-quarter of the amount of Cripto was expressed compared to the controls (Figure 3). Thus, the retroviral vector constructs effectively modulated Cr-1 expression in CID 9 cells. Both cytokeratin positive and vimentin positive cells were observed, using indirect immunofluorescent staining, in all the populations. The proportion of keratin-positive cells was similar in all three populations, amounting to $55 \pm 10\%$. Thus, there was no selection of one population and all the cell lines contained the two distinct cell types described by Desprez (Desprez *et al*, 1993).

Morphology

Monolayer cultures of CID 9 cells commonly show two cellular morphologies, a spindle-shaped and a typical epithelial cobblestone pattern (Figure 4A). No differences were observed in the mixed morphology of cells after infection with the empty vector compared to uninfected CID 9 cells (Figure 4A and B). The cells infected with the sense Cripto cDNA also exhibited the same cellular morphology as the CID 9 cells or the pGCEN control CID 9 cells at both high and low cell densities. No significant morphological differences were observed during continuous propagation of these various pools. At low density, cells grew as islands of cells. In cultures remaining at confluence for 2 days, the presence of domelike structures was apparent. Cr-1 overexpressing cells formed more frequent and larger domelike structures (Figure 4C) compared to wt cells. Cells expressing antisense Cripto became morphologically distinct; the individual cells were flatter and spread to a larger area at both high and low density. They showed the more typical cobblestone morphology of epithelial cells (Figure 4D) and at confluence, they did not form domes. However, like the parental cells, they still contained at least two cell types that expressed keratin or vimentin intermediate filaments (Desprez *et al*, 1993), and did not represent a specifically selected population.

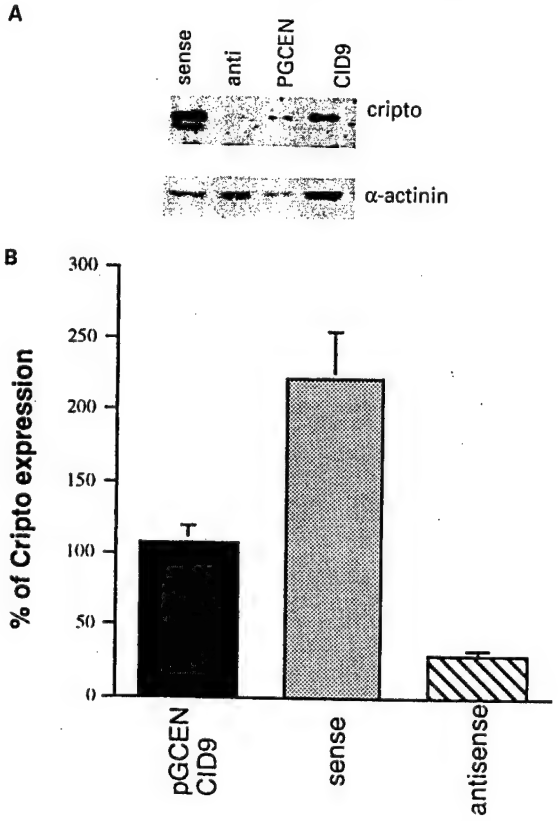

Figure 3 Cripto expression levels in infected CID 9 cells. (**A**) Overexpression of Cripto in CID 9 cells infected with the pGCEN-sense Cripto vector (sense) and underexpression in cells infected with the pGCEN-antisense Cripto vector (anti). α-Actinin was used to show relative protein levels in each lane. (**B**) Graph of the percentage of Cripto expression ± S.D. in two different populations of infected cells compared to control pGCEN vector infected CID 9 cells (pGCEN) or uninfected CID 9 cells (CID 9) which was defined as 100%

Cell proliferation and apoptosis

To determine if over- and under-expression of Cr-1 had an effect on mammary cell growth, several types of growth assays were performed. Cell growth rates were determined using maintenance culture medium. In this media, the CID 9 cells overexpressing Cripto (sense), underexpressing Cripto (antisense), and the control cells containing the empty vector (pGCEN) all grew at approximately the same rate for the first 24 h. Then the cells overexpressing Cripto grew faster so that at 48 and 72 h there were a greater number of cells compared to the control. The cells with reduced Cripto appeared to reach confluence by 24 h and the level of cells subsequently decreased after 48 h suggesting that they were starting to die (Figure 5A).

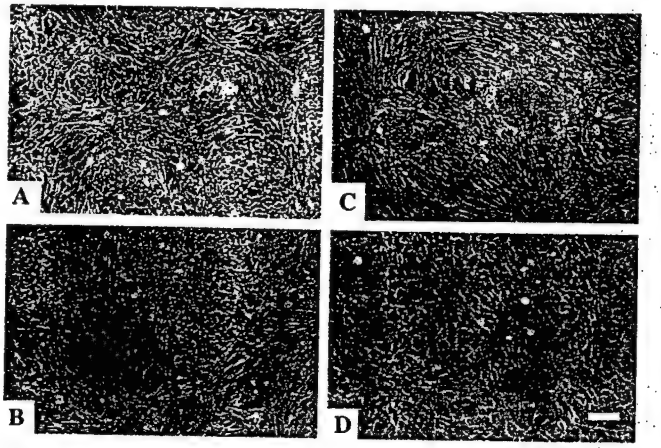

Figure 4 Morphological effect of Cripto over- and under-expression on CID 9 cells. Phase contrast micrographs compare the general appearance of the (**A**) normal CID 9 cells with (**B**) cells infected with control pGCEN vector, and (**C**) cells infected with the Cripto expression vector, and (**D**) cells infected with the antisense Cripto expression vector. All the cells were seeded at the same density and allowed to grow the same length of time. For all panels the bar indicates 100 μm

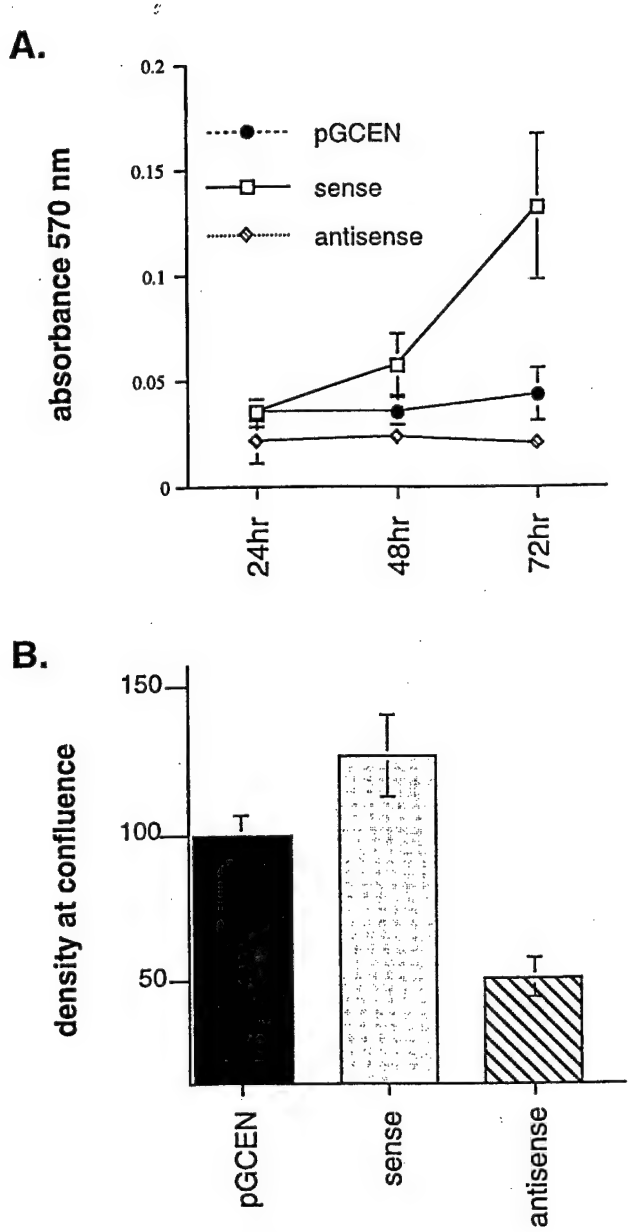

Figure 5 Cell growth assays. Growth rates of CID 9 cells containing the empty vector (pGCEN), the Cr-1 expression vector (sense), and the antisense Cr-1 expression vector (antisense). (**A**) The absorbance at 570 nm is directly proportional to the number of cells/well. (**B**) Plateau growth density of cells expressing various levels of Cr-1. Values are expressed as a percentage of the CID 9 cells containing the control pGCEN vector. Bars, S.D.

To determine cell densities at confluence, cells were grown in 2% fetal bovine serum (FBS), allowed to grow for 5 days and remain at confluence for 2 days and then counted (Figure 5B). The Cr-1 overexpressing cells grew to a greater density (3.2×10^5 cells/cm^2) than the control cells (2.4×10^5 cells/cm^2). For CID 9 cells expressing decreased levels of Cr-1, the density at confluence remained at 1.2×10^5 cells/cm^2. Thus the cells containing the antisense vector showed contact inhibition at a statistically significant ($P<0.05$) lower cell density compared to parental and control cells.

To determine if Cr-1 lowered the requirement of CID 9 cells for growth factors, cell proliferation assays were also performed by plating the cells in media containing 2% FBS to allow attachment and then growing them in serum-free conditions. Uninfected CID 9 cells and cells containing the sense or empty vector grew at approximately the same rate and all the rates were lower than in 2% or 5% FBS conditions. The CID 9 cells containing the retrovirus with Cr-1 in the antisense orientation showed no growth in serum-free media and after several days no cells survived (data not shown).

This observation suggested that the loss of Cr-1 expression might affect survival of cells even in serum-containing cultures and this was tested next. Apoptosis levels were assessed by nuclear morphology and by TUNEL assays (Figure 6a). Numbers of apoptotic nuclei (Figure 6b) in log phase CID 9 cells were $0.60\% \pm 0.20$ of the population in control pGCEN cells; this rate decreased to $0.28\% \pm 0.16$ ($P<0.05$) in Cripto overexpressing cells and increased to $1.53\% \pm 0.66$ ($P=0.035$) in antisense cells. More frequent apoptotic nuclei were observed in antisense-expressing cells (Figure 6a, E, F) than in either of the other two cell populations (Figure 6a, A, B, C, D). The expression of Cripto therefore endows a growth advantage as well as better survival in cultured mammary cells.

Colony formation and anchorage independent growth

The Cripto overexpressing sense CID 9 cells have a greater tendency to build up multilayers of cells in monolayer cultures compared to control pGCEN infected or uninfected CID 9 cells (Figure 7A and B). The normal cells formed colony-like domes but CID 9 cells underexpressing Cr-1, on the other hand, showed very little colony formation compared to control cells and ceased to proliferate after reaching confluence (Figure 7C). The three CID 9 populations showed differential abilities to grow in soft agar (Figure 7). Control cells formed 15 ± 3 colonies per dish or 0.04% of the plated cells formed colonies (Figure 7D). Whereas the Cripto overexpressing cells formed greater than 79 ± 6 colonies or 0.2% of plated cells formed colonies (Figure 7E). The antisense cells did not grow in soft agar and were therefore anchorage dependent for growth (Figure 7F).

Differentiation

CID 9 cells differentiate efficiently on an extracellular matrix in the presence of lactogenic hormones. Our three populations, containing either the sense, antisense, or empty pGCEN vector, were allowed to differentiate on Matrigel™, where morphological differentiation can be observed by the ability to form polarized epithelial structures termed mammospheres. The empty pGCEN infected CID 9 cells appeared the same as the parental CID 9 cells in that they formed frequent mammosphere structures (Figure 8A). The CID 9 cells overexpressing Cr-1 grew faster than the control cells (data not shown) and there were overlapping cell layers, however, no mammosphere structures formed (Figure 8B). This suggests that overexpression of Cr-1 in cells overrides the

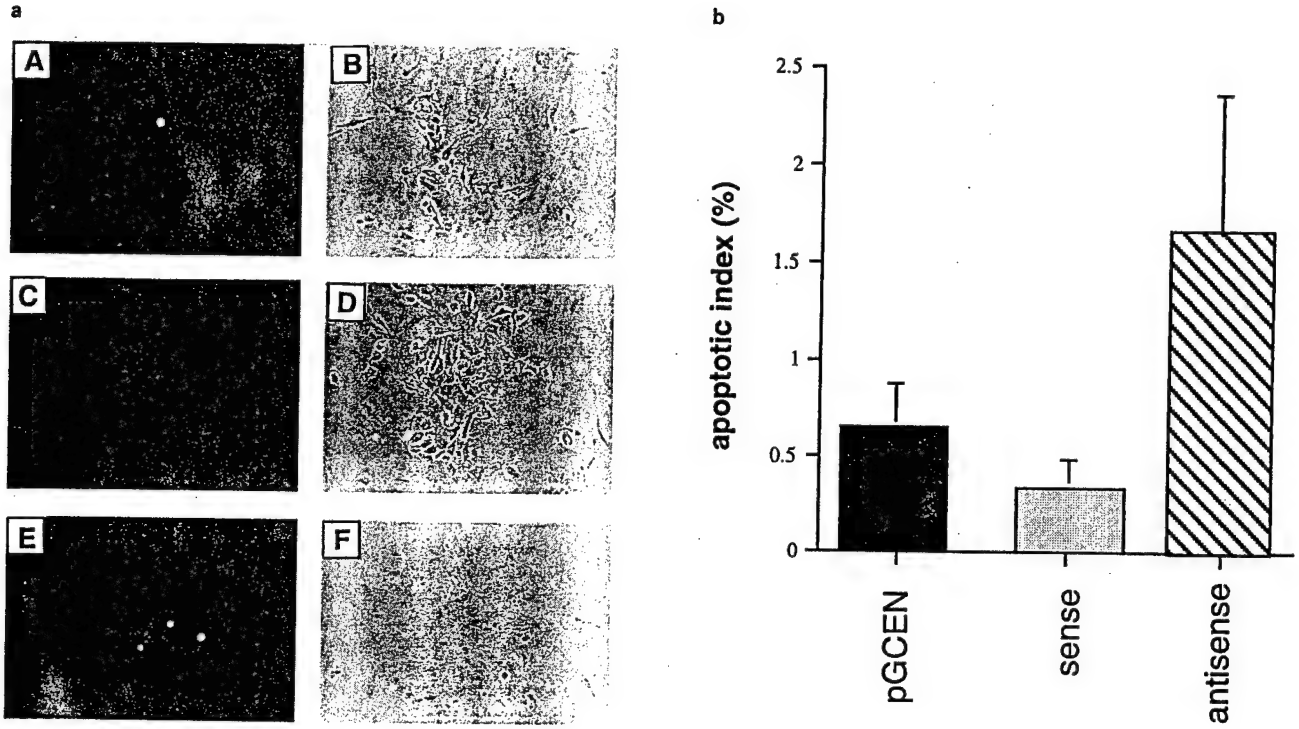

Figure 6 The effect of Cr-1 on CID9 cell survival. (**a**) *In situ* detection of apoptotic cells with the TUNEL method (left panels, A, C, E) and phase contrast microscopy (right panels, B, D, F) of CID 9 cells expressing various levels of Cr-1. All cells were plated at the same density and grown under the same conditions. A, B, control cells containing the empty pGCEN vector; C, D, CID9 cells overexpressing Cr-1 (sense); E, F, cells underexpressing Cr-1 (antisense). One representative field of each population is shown. (**b**) Apoptotic levels of log phase CID 9 populations (see Materials and Methods for details). Bars, S.D.

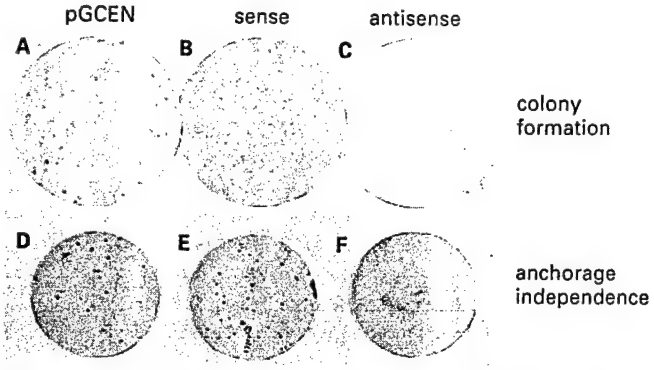

Figure 7 Colony formation in monolayer culture and anchorage independence (soft agar) assays of various populations. The CID 9 cells containing the empty vector (pGCEN) show some colonies formed both on plastic (**A**) and in soft agar (**D**). CID 9 cells overexpressing Cr-1 (sense) show a threefold greater number of colonies both on plastic (**B**) and in soft agar (**E**). Whereas, CID 9 cells underexpressing Cr-1 (antisense) show very little or no colony formation on plastic (**C**) or in soft agar (**F**)

signals leading to differentiation that normally appear. Interestingly, antisense expressing cells also did not form any mammospheres but tended to aggregate into aster-like structures (Figure 8C).

Biochemical indications for differentiation in mammary cells are the syntheses of milk proteins including β-casein. The CID 9 cells were grown in the presence of the lactogenic hormones, insulin, prolactin, and hydrocortisone either on an extracellular matrix or on plastic, and β-casein was detected by immunoblotting with an antibody to mouse milk proteins. Equal loading of gels was verified by immunoblotting with an antibody to α-actinin (Figure 9, lower panel) and the levels of Cr-1 produced in the cells collected after the experiment was confirmed by immunoblotting with anti-Cr-1 (Figure 9, upper panel). The CID 9 cells overexpressing Cr-1 showed only about half the amount of β-casein expression compared to the control CID 9 population (Figure 9, lanes 2, 3, 11, and 12). This agrees with our morphological observations that Cr-1 overexpressing cells are predominantly proliferating compared to the control or normal CID 9 cells. Unexpectedly, the antisense containing CID 9 cells showed equal or greater expression of β-casein compared to the control suggesting (a) that Cr-1 is not involved in β-casein expression and (b) that mammospheres are not important for differentiated expression. The addition of matrix is not necessary for casein expression (Figure 9, lanes 10–12), probably because CID9 cells synthesized their own matrix during this time. All populations of CID 9 cells (sense, antisense or empty vector) if grown in maintenance media without hydrocortisone and prolactin, either to confluence or not, failed to express β-casein (Figure 9, lanes 4–9).

A milk protein of approximately 22 kDa whose regulation was distinct from the control of β-casein expression (Marte *et al*, 1995a) was produced by the CID 9 cells. Unlike β-casein expression, this protein was expressed in the cells whether lactogenic hormones were present or not. This

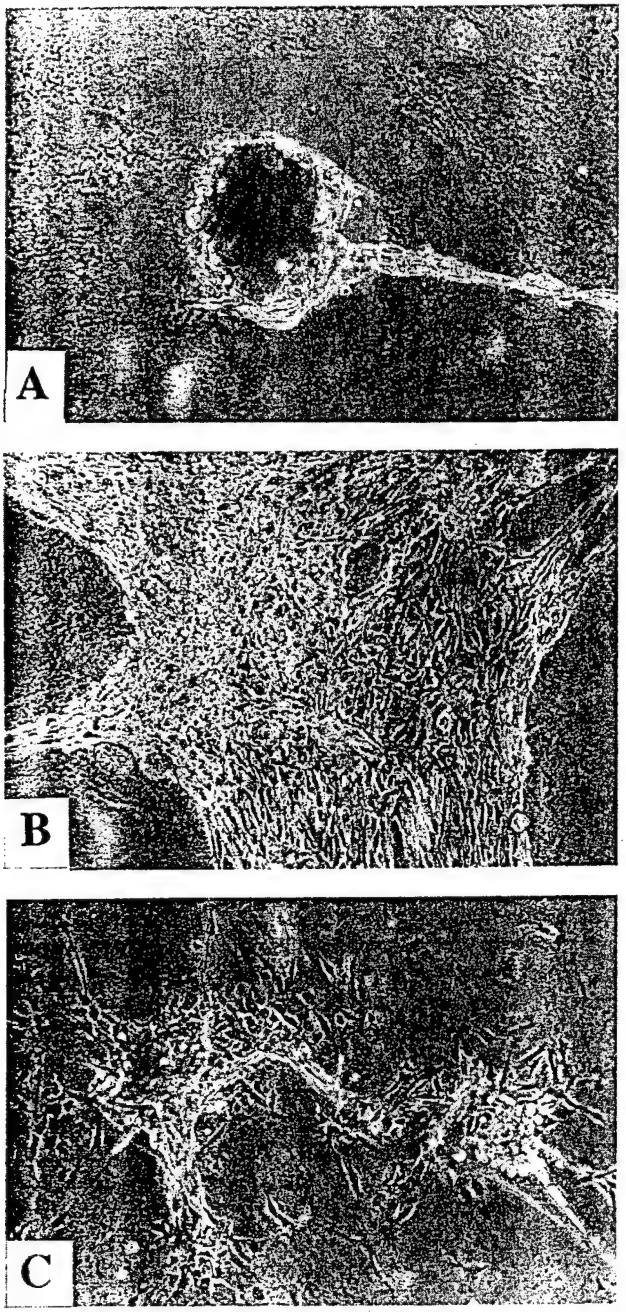

Figure 8 Morphological appearance of CID 9 cell populations grown on extracellular matrix (Matrigel) in the presence of lactogenic hormones. Phase contrast micrographs showing (**A**) normal differentiated structures (mammospheres) formed in control population of CID 9 cells containing the empty pGCEN vector, (**B**) overgrowth of CID 9 cells overexpressing Cr-1, no mammosphere structures were observed and (**C**) aster-like structures formed when decreased levels of Cripto were expressed. All the cells were seeded at the same density and allowed to grow for 6 days

protein was expressed at approximately the same level in all cell populations except Cr-1 overexpressing CID 9, which expressed only 15% of the level of expression of the 22 kDa protein observed in the other populations (Figure 9, lane 2). This property further distinguished the Cr-1 overexpressing cells.

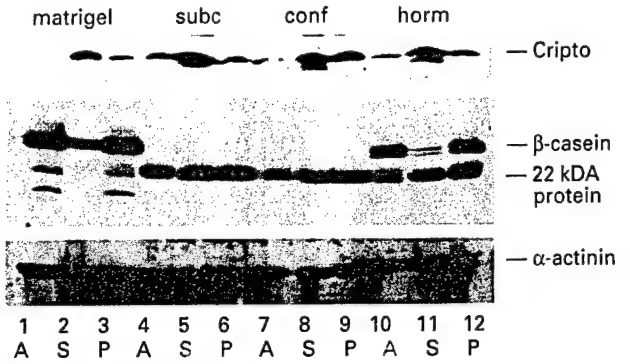

Figure 9 Immunoblots to show proteins produced by populations of CID 9 cells grown under various conditions. CID 9 cells underexpressing Cr-1 (**A**), overexpressing Cr-1 (S) or expressing normal levels of Cr-1 and containing the empty vector (P) were analyzed. Growth conditions included growth on Matrigel in the presence of lactogenic hormones for 6 days (Matrigel, lanes 1–3); growth on plastic in normal maintenance media until they were still subconfluent for 3 days (subc, lanes 4–6); growth on plastic in normal maintenance media until they were confluent for 5 days (conf, lanes 7–9); and growth on plastic in the presence of lactogenic hormones for 6 days (horm, lanes 10–12). The top panel indicates the Cripto expression in the populations. In the middle panel to compare the differentiation of cell populations, the 26 kDa β-casein and the 22 kDa milk proteins are indicated. α-Actinin staining was used as a control for equal loading of protein in the bottom panel

CID 9 cells *in vivo*

To determine if the over- or under-expression of Cr-1 had an effect on mammary gland development *in vivo*, we transferred CID 9 cells into the cleared fat pads of Balb/c mice. Unexpectedly, both Cr-1 sense-infected and uninfected CID 9 cells formed tumors in the transgenic fat pads, 5 to 6 weeks after transfer. We also tested the tumorigenic potential of the antisense Cripto expressing CID 9 cells. They remained tumorigenic, giving rise to tumors 6 weeks after insertion into the mammary fat pad of syngeneic Balb/c mice. The tumor growth rates were similar and the cells were histologically indistinguishable. Control mammary glands that were sham operated remained normal. Western blot analysis showed that approximately one-fifth the amount of Cripto was expressed in the tumors formed from antisense Cr-1 CID 9 cells compared to the parental CID 9 cells (data not shown). Therefore loss of antisense effect could not explain the unchanged tumorigenic potential of the CID 9 cells *in vivo*. We concluded that CID 9 cells were tumorigenic for reasons not relevant to Cripto expression.

We therefore turned to the parental cell line of the CID 9 cells, Comma-1D cells, to perform the same study, predicting that these cells may not be tumorigenic. Although uninfected Comma-1D cells transferred to cleared mammary fat pads produced no tumors, they did form hyperplastic outgrowths at the injection site in agreement with previous studies (Aguilar-Cordova *et al*, 1991). Comma-1D cells infected with the empty pGCEN or with the Cripto-expressing vector also formed hyperplastic outgrowths of similar sizes in numerous cases for each cell line. In contrast, Comma-1D cells infected with antisense Cr-1, were unable to grow in cleared fat pads, supporting

the finding that loss of Cr-1 decreases cell survival. This was retested in primary mammary cultures that were antisense Cr-1-infected and these also would not repopulate the cleared mammary fat pad, whereas uninfected cells would do so.

Discussion

The mammary gland is a highly complex system of interacting cell types. At birth, it contains at least three epithelial compartments: luminal epithelium, alveolar epithelium, and myoepithelium (Streuli *et al*, 1995). The mammary gland goes through four distinct postnatal developmental stages. Estrogen dependent ductal growth occurs from 5 to 8 weeks of age in the mouse when epithelial 'end buds' ramify from the nipple throughout the fatty mesenchyme creating a bush-like network of ducts. The onset of pregnancy initiates a second phase of extensive proliferation in which lobular-alveolar structures develop from the existing ductal system, in a process that is driven by pregnancy-induced hormones and estrogen (Nandi, 1958). It was from this stage that CID 9 cells were derived. The lobular system grows and differentiates to form alveoli in which milk protein synthesis occurs during lactation, the third phase of development. Following weaning of the young, the mammary gland undergoes extensive remodeling, leading to the loss of the alveolar structures, a process called involution involving large scale apoptosis (Strange *et al*, 1995). Each phase of mammary gland development requires a specific combination of systemic hormones that presumably activate different combinations of locally acting factors. Signaling molecules that have been implicated in local actions include members of the EGF, Wnt, FGF and TGF-β families (Coleman and Daniel, 1990) (Snedeker *et al*, 1991). Each gene is expressed differentially during these developmental stages. We show here that Cr-1 is one of this group of genes, being strongly active during pregnancy, less active in lactation and switched off during involution of the mammary gland after pregnancy. This suggests both that Cr-1 expression is regulated by pregnancy hormones and that Cripto has a function during these stages of mammary gland development.

In this study we took advantage of a mammary cell subpopulation that mimics pregnant mammary glands in vivo. CID 9 cells allowed us to assay both growth and differentiation events in culture. We found that Cr-1 was expressed at higher levels when cells were stimulated to grow and differentiate to lactogenic phenotypes (in the presence of lactogenic hormones and extracellular matrix), similar to pregnant glands *in vivo* (Figure 1). This strengthens the hypothesis that these hormones up-regulate Cr-1 expression as they do amphiregulin (Martinez-Lacaci *et al*, 1995) and TGFα (Kenney *et al*, 1993; Reddy *et al*, 1994) in mammary tissue.

Kenney *et al.* (1995) showed by RT–PCR, the presence of Cr-1 mRNA in the virgin mouse mammary gland, although 24 or 26 kDa proteins were not observed using an anti-human Cr-1 antibody. We were able to detect low amounts of a Cr-1 protein of 26 kDa in virgin glands of 8 week and older mammary glands (Figure 1, lane 1) using a mouse-specific Cr-1 antibody. Twenty-four kDa is the predicted and expected size of the mouse secreted Cripto protein, demonstrated by Brandt *et al.* (1994) to be authentic Cr-1. In our analyses using immunoblotting of mammary gland tissues and cell lysates, we detected two Cr-1 proteins of 24 and 26 kDa (Figure 1) which are likely glycosylated or myristylated differently (Brandt *et al*, 1994). Cr-1 protein is expressed in undifferentiated F9 embryonal carcinoma (EC) cells as a protein of 24 kDa (Figure 1, lane 5) but is down-regulated in differentiated F9 cells, in agreement with others (Ciccodicola *et al*, 1989). CID 9 cells differentiate *in vitro* and we showed that Cr-1 has an inhibitory influence on differentiation. We demonstrated that proliferation increased and cell death decreased in CID 9 cells overexpressing Cr-1. Two separate assays, mammosphere formation and milk protein expression, showed that overexpression of Cr-1 led to decreased differentiation. In agreement with our data, a 47-mer, containing the EGF-like motif only of recombinant human CR-1 is able to stimulate proliferation and inhibit β-casein expression in mouse HC11 mammary epithelial cells (Kannan *et al*, 1997). It is generally accepted that proliferation rates are inversely related to differentiation, and we demonstrated here that Cr-1 stimulated the proliferation needed to bring the cells to the state required (equivalent to pregnancy) for differentiation (lactation) but inhibited the differentiation process itself.

We identified β-casein as a major product in differentiated CID 9 cells. Another milk protein of 22 kDa was also observed to be regulated independently of β-casein, a finding similar to that of Marte *et al*, (1995b) in neuregulin-treated HC11 cells which were also derived from COMMA-1D cells. The 22 kDa milk protein appeared to be specifically down regulated in CID 9 cells overexpressing Cr-1 when grown on an extracellular matrix. The nature of this protein and the mechanism of its regulation remains unknown.

How does the evidence presented here fit with the suggestion that Cr-1 is an oncogene? Cripto overexpression caused the cells to grow faster, to become less contact inhibited, allowed growth in domes, caused cells to survive clonal cell growth, and allowed anchorage independent growth (Figure 5, 6 and 7). Together these results suggest that Cr-1 overexpression leads to a transformed phenotype *in vitro* that would characterize Cr-1 as an oncogene. Moreover, when Cr-1 expression was reduced in cells that normally expressed it, they grew at one-third the rate of the control cells and became contact inhibited earlier and failed to grow anchorage independently or at clonal densities. This clearly suggests that Cr-1 is an autocrine growth factor for CID 9 cells. Our data and the NOG-8 results (Ciardiello *et al*, 1991) support the hypothesis that overexpression of Cr-1 leads to transformation and increased proliferation of normal mouse mammary epithelial cells. Our results also show that Cripto is required for normal growth and morphology of mammary cells during the pregnancy stage.

In contrast, *in vivo* tests do not lend support to a tumorigenic activity of Cr-1 in mammary cells. Although CID 9 cells are an excellent model for 'normal' mammary epithelial cell growth and differentiation *in vitro*, they proved to be tumorigenic when transplanted into syngeneic cleared

fat pads. In fact, control CID 9 cells (infected with empty vector) were able to grow in soft agar, suggesting they were already transformed. COMMA-1D cell studies were somewhat more revealing because when infected with the antisense Cr-1 retroviruses, they were unable to grow at clonal densities, indicating that these cells are much more sensitive to apoptosis in the absence of Cr-1. It is informative that NOG-8 mouse mammary cells even when over-expressing CR-1 do not form tumors in nude mice (Ciardiello *et al*, 1991), also suggesting that Cr-1 is not a tumor-inducing gene.

Some questions about Cr-1 need further study: What is the receptor for Cr-1 in CID 9 cells? What is the precise role of Cr-1 in human breast carcinoma, could Cr-1 act as a survival factor that allows the cells to escape apoptotic signals? High expression of Cr-1 in mouse and human embryonal carcinoma cells suggests that Cr-1 could play a role in teratocarcinoma formation (Baldassarre *et al*, 1997).

In summary, the results suggest that Cr-1 is positively regulated by lactogenic hormones and is important for mammary cell proliferation during the pregnancy stage of mammary gland development. Its function during pregnancy in the mammary gland may include differentiated cell renewal and survival but it is not directly involved in differentiation. Cr-1 also causes transformation *in vitro* and increases the rate of cell growth in mammary cells, a role suspected earlier because of its prevalent expression in breast and colon tumors. Using both *in vitro* and *in vivo* model systems we are addressing some of the questions concerning the regulation of Cr-1 and more specifically its role in mammary gland development and cancer.

Material and Methods

Cell culture

CID 9 cells were kindly provided by Dr. Mina Bissell (Lawrence Berkeley Laboratory, Berkeley, CA). COMMA-1D cells were provided by Dr. Dan Medina (Baylor College of Medicine, Houston, TX). Cells were maintained in 1:1 DMEM:Hams F12 Nutrient Mixture (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FBS and insulin (5 μg/ml). For differentiation, the cells were grown for 7 days in the presence of lactogenic hormones (insulin 5 μg/ml, hydrocortisone 1 μg/ml, and prolactin 3 μg/ml) on Matrigel (Collaborative Biomedical Products, Bedford, MA). The cells were plated in 1:1 DMEM:F12 Nutrient mixture, 2% fetal bovine serum (FBS), and lactogenic hormones at a density of 6×10^4 cells/cm^2. After 24 h the dishes were washed twice with PBS and fed with media containing no FBS but with lactogenic hormones.

Cell proliferation was quantified by determining formazan production from tetrazolium salt using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) and a ELISA plate reader programmed to quantify absorbance at 570 nm and background at 630 nm. All assays were performed under conditions where the 570 nm absorbance readings were directly proportional to the number of cells/well. The anchorage independent growth assays were performed using a modification of a method described by Stoker *et al*, (1968). Cells were plated at a density of 4×10^4 cells per well of a 6 well plate in culture medium supplemented with 0.4% noble agar (Difco, Detroit, MI) over a lower layer of 0.3% agar, and allowed to grow for 21 days. Colonies were stained overnight with 0.05% p-iodonitrotetrazolium violet (Sigma Chemical Corp., St. Louis, MO), a vital stain that is taken up by mitochondria in cells.

Programmed cell death was quantified by (a) counting the the number of fragmented nuclei after staining logarithmically growing cultures with bisbenzamide (Sigma) and (b) *in situ* labeling of apoptosis induced DNA strand breaks (TUNEL assays) as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Greater than 2000 cells were examined per assay and each assay was repeated twice. The results were combined to give the results in Figure 6B.

In these assays, statistical analysis in the Macintosh Excel program was applied, using the students two-tailed *t* test.

Infections

The pGCEN vector was kindly provided by Dr. Richard Morgan (National Institutes of Health, Bethesda, MD) (Figure 2). The pGCEN neo retroviral vector expresses an inserted gene from the Moloney murine leukemia virus Long Terminal Repeat (LTR). An IRES sequence allows the expression of the neomycin resistance gene from the same promoter (Boris-Lawrie and Temin, 1993). The sense and antisense Cripto cDNA clones containing full length murine Cr-1 coding sequence was inserted into the *Xho*I site of the polylinker region in both sense and antisense orientations. PA317 producer cells (Miller and Buttimore, 1986) were transfected with 20 μg vector coprecipitated with calcium phosphate. Positive clones were selected with 800 μg/ml G418 after 5 days in culture. Supernatants free of G418 were collected and used immediately or aliquoted and stored at -70°C.

The CID 9 cells were infected with retroviral supernatant containing Cr-1 either in the sense or antisense orientation or the empty vector. The cells were plated at 8×10^5 cells per 100 cm^2 dish and the next day fed with 8 ml of viral supernatant (prefiltered through a 0.45 mm filter), 8 ml Hams F12 media, 4 μg/ml polybrene and 5 μg/ml insulin. After 24 h the infected cells were selected using G418 (400 μg/ml) in the media. Greater than 250 colonies were pooled and used in the studies as a mixed population. Because both Cr-1 sense and antisense sequences and the selectable marker neo are expressed from a single promoter using this vector, the cells were tested every few passages to determine that they maintained G418 resistance and hence Cripto expression.

Expression analysis

Mammary protein was obtained from mammary glands of staged mice. The tissues were homogenized in hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 1 mM $MgCl_2$; 1 μg/ml phenylmethylsulfonyl fluoride; 20 μg/ml aprotinin) and solubilized in sample buffer (Laemmli, 1970). Culture dishes were washed twice in phosphate buffered saline (PBS) and lysed in sample buffer. Cells grown on the extracellular matrix were first treated with dispase (Collaborative Biomedical Products, Bedford, MA) for 1 h to dissolve the matrix and then washed and lysed as above. Equal amounts of protein were electrophoresed on a 15% SDS–PAGE gel and electrotransfered to Immobilon membranes (Millipore Corporation, Bedford, MA). Western blot analysis was performed and visualized using the ECL detection system (Amersham Corp., Little Chalfont, UK). Indirect immunofluorescence assays were done on fixed cell monolayers using monoclonal antibodies to pan-keratin or vimentin (Sigma Chemical Corp., St Louis, MO) with FITC-labeled rabbit anti mouse Ig as secondary antibodies.

The rabbit polyclonal antibody was raised against a murine Cripto peptide, amino acid sequence 26 to 39, RDLAIRDNSIWDQK. The antimouse milk serum was a generous gift from Dr N Hynes (Friedrich Miescher Institute, Basel Switzerland). This antiserum recognizes several milk protein including β-casein and the 22 kDa protein (Marte *et al*, 1995b). Sheep anti-mouse casein antibody was kindly supplied by Dr B Vonderhaar (NCI). A rabbit polyclonal antibody to rat α-actinin was a gift from Dr J Singer (UC San Diego, CA) and served as a control for equal protein loading on the gel.

Mammary fat pad transplants

Mice were anesthetized with avertin. Mammary gland 'clearing' was performed on the right inguinal #4 fat pads of mice at 3 to 4 weeks of age as described previously (Deome *et al*, 1959; Faulkin and Deome, 1960). In essence, the nipple and primitive adjacent mammary epithelial tissue were excised while the remaining fat pad provided the region for growth of transplanted cells. The CID 9 cells were washed in serum-free medium and injected into the fat pads at approximately 1×10^5 cells per fat pad in 5 to 10 μl volumes (Edwards *et al*, 1988). The skin was sutured, and mice maintained for 6 weeks to allow the transplanted cells to grow in the fat pad. Glands or tissues were dissected out, divided into portions and frozen for later analysis.

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Canc Res-Adv in brief

Preneoplastic mammary tumor markers: Cripto and Amphiregulin are overexpressed in hyperplastic stages of tumor progression in transgenic mice

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Abstract

Amphiregulin (Ar) and Cripto-1 (Cr-1) are autocrine growth factors for mammary cells and both have been observed to have high expression in human mammary tumors, in contrast to adjacent tissues. To investigate whether Ar and Cr-1 play roles in the progression of mammary cell proliferation to unregulated growth and tumor formation, the levels of expression were examined in transgenic mice (TGM) that over-express several different oncogenes: MMTV-Polyoma virus middle T antigen (MMTV-PyMT); MMTV-c-ErbB2 (c-neu, HER2); and MT-hTGFα. These transgenic mice all produce mammary tumors but with different rates of progression. The levels of Ar were induced up to 10-fold in association with hyperplasia in two of the TGM. Cr-1 overexpression was consistently observed in hyperplastic mammary glands in all the animal models, decreasing in overt tumors in 2 of the TGM models. In MMTV-PyMT mammary glands, the levels of Cr-1 expression rose 7 to 10-fold in hyperplastic tissue and 25-fold the levels in tumors compared to age-matched transgene negative mice. Ar and especially Cr-1 have potential value as markers of preneoplastic change in mammary tissue.

Introduction

A major goal of cancer research is to identify transforming oncogenes and determine their precise role in tumor development. Genes such as Amphiregulin (*Ar*), Transforming growth factor alpha (*TGFα*), Cripto-1 (also known as *TDGF-1*), Epidermal growth factor receptor (*EGFR*) and *ErbB2/neu* are over-expressed in, and/or are markers for, human breast carcinomas. Invasive mammary carcinomas generally exhibit increased levels of Ar protein and/or mRNA compared to ductal carcinomas *in situ* or normal mammary epithelium. Ar, one of several ligands for EGFR, is believed to act as a mediator of proliferation and/or differentiation via an autocrine mechanism (1). Of 68 biopsies on breast carcinomas, 82% expressed CR-1 (2). CR-1 (receptor not known) expression was not detected in normal human breast tissue or cell lines, and its appearance was thought to be associated with transformation. We have recently shown that Cr-1 is highly expressed during the pregnancy and lactation stages of normal mouse breast development acting as both a proliferation and survival factor (3) (4). About 40% of breast tumors over-express epidermal growth factor receptor (EGFR) (5). EGFR is a transmembrane inserted tyrosine kinase protein with a Mr of approximately 170,000 (6) that can be activated by the ligands EGF, TGFα, betacellulin and epiregulin in addition to Ar. The second member of the ErbB family of receptor genes, *ErbB2* gene (*HER2, c-neu*), encodes an Mr 185,000 transmembrane tyrosine kinase protein (7) (8). Amplification and overexpression of the ErbB2 has been observed in nearly 30% of human cancers, particularly intraductal carcinomas (9).

In this study we used transgenic mice overexpressing PyMT, erbB2, or TGFα in the mammary glands (10-12) to study the expression patterns of two recently discovered growth factor breast cancer markers, Ar and Cr-1. The present report indicates that Ar and Cr-1 could be useful as early markers of hyperplastic growth that precede the onset of tumors.

Materials and Methods

Western blot analysis: Mammary tumors and tissues were obtained from staged mice. The tissues were homogenized in hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 1 mM $MgCl_2$; 1 μg/ml phenylmethylsulfonyl fluoride; 20 μg/ml aprotinin) and solubilized in Laemmli sample buffer. Equal amounts of protein were electrophoresed on a 15% SDS-PAGE gel and electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Western blot analysis was performed and visualized using the ECL detection system (Amersham Corp., Little Chalfont, UK).

Antibodies: The Cr-1 antibody used in this study is a generous gift from M.G. Persico. It consisted of a rabbit polyclonal raised against a murine Cripto peptide, amino acid sequence 26 to 39, RDLAIRDNSIWDQK. The amphiregulin antibody used in this study, AB2426, was a rabbit polyclonal raised against a synthetic peptide corresponding to the murine amphiregulin sequence 119 to 134, RKKKGGKNGKGRRNKK. The ErbB2 antibody was obtained from Santa Cruz Biotechnology, and sheep anti-human EGFR intracellular domain was from Gibco-BRL.

Transgenic mice: The PyMT mice (in an FVB/N background) have been fully described previously (13). MMTV-neu mice (JR2376; FVB/N-TgN(MMTVneu)202Mul) were obtained as homozygotes also in an FVB/N background from The Jackson Laboratory (12). MT-TGFα transgenic mice were described earlier (10, 11). Transgenic lines were maintained in The Burnham Institute Animal Facility by mating founder/positive animals to littermates. Transgenic mice were identified by polymerase chain reaction (PCR) analysis of tail DNA using oligonucleotide primers specific for the transgene. For each stage, the left 4th inguinal mammary

gland was dissected out for immunoblotting and the right gland was stained as a whole mount, to indicate the tumor status by gross morphology. Hyperplasia was recognized by numerous small multifocal H & E-staining islands of ductal or lobular epithelial cell proliferation, occupying □ 20% of the total gland. Larger dense aggregates of cells were designated tumors, of which the largest were palpable. Two or three samples were used for each data point, except for MT-TGFα mice where there were 2 tumors and one of each of the other samples.

Results

MMTV-PyMT transgenic mice (TGM)

PyMT overexpressing FVB/N TGM express the oncogenic polyoma virus middle T antigen from the MMTV promoter/enhancer. Extensive multifocal transformation of the mammary epithelium ensues and rapidly progresses to mammary adenocarcinomas (13). Hyperplasias start to appear on the 32nd day in females, and therefore mammary glands from virgin females were examined at postnatal days 20, 28, 32, 44 and 56 days. We studied 2-3 animals per sample point and found no palpable tumors up to day 44, but by day 56, palpable tumors were present. Immunoblot analyses to detect and quantify the levels of Ar and Cr-1 proteins in mammary tissues together with EGFR and ErbB2, were made (Fig. 1). Densitometry measurements of the results were normalized for the protein content by the signal obtained from either β-actin or α-actinin on all analyses. The highest level of each gene product was given a score of 100% and the other samples were compared to this level in Table 1. For comparison, normal tissues and tumors were retrieved from both transgene negative and positive 8 week virgin mice, respectively; 13.5 day pregnant mice and one mouse that was one day postpartum, and was not able to lactate.

Amphiregulin was expressed in the mice positive for the PyMT transgene (+ in Fig. 1) in mice 28 and 32 days old but not in normal littermates (-) at these timepoints. Interestingly, at day 20, when sex steroid hormones were being activated at the onset of puberty, Ar expression was observed in both positive and negative mice, and was 1.5-fold higher in the transgene positive mice. By day 44 hyperplasia and scattered non-palpable dense aggregates were observed (data not shown) and Ar levels increased significantly in this tissue (Fig. 1, lane 10). Ar levels were 10-fold increased in hyperplastic glands, and up to 25-fold higher in tumors of 56-day virgin mouse mammary glands (Table 1). However, there was no significant difference between the Ar expressed by pregnant Tg+ or Tg- mice or by lactating mice. These hormone-induced levels may be the highest possible that can be expressed by the mammary gland and was given a score of 100% in Table 1. Among the different isoforms of Ar that were seen in these studies, only a large doublet of 28 and 25 kDa were produced by the PyMT TGM mammary glands.

Using the same lysates, no Cr-1 expression was observed until day 32 and these were very low levels that were no greater than in the control (-) mouse. We have observed before that Cr-1 is expressed at very low but detectable levels in older virgin mice (Fig.1, lanes 5,7,8) (4). By day 44 in virgin females, Cr levels were 7- to 10-fold higher than in control (-) mice (Fig. 1A, lane 10). At 56 days of age, the tumors from virgin mice showed 25-fold higher levels of Cr-1 than the transgene-negative samples (data not shown). The tumors in pregnant and lactating mice expressed high levels of Cr-1 that were not significantly different from the levels of Cr-1 expressed in the non-transgenic mice (Table 1). This is probably due to the increased levels of prolactin and glucocorticoids that upregulate Cr-1 (and Ar) during pregnancy (4). Several

isoforms of Cr-1 were observed (28, 27, 25 kDa), probably caused by differential post-translational modifications and by degradation in larger tumors that were necrotic, and have described previously by others (3, 14, 15).

The levels of EGFR protein expressed in all of the samples derived from PyMT positive and normal mammary glands were low and unchanging through the progressive series except for a slight up-regulation at puberty (Fig. 1A, lanes 3 to 6). No differences were observed between tumors derived from virgin, pregnant, and lactating mammary glands. ErbB2 protein was observed in all of the MMTV-PyMT mammary glands and tumor samples assayed, but there was no significant difference between mice that were positive for MMTV-PyMT and normal mice until the time that hyperplasia was seen (Fig. 1, lanes 9, 10). Thereafter tumors expressed 2- to 3-fold more ErbB2 than their non-transgenic sisters (Table 1).

MMTV-ErbB2 transgenic mice

Overexpression of the normal c-ErbB2 gene gives rise to mammary tumors with a long latency (12). According to Guy *et al*, (1992), tumors first appear as foci in surrounding hyperplastic growth at or after 4 months of age with a median incidence of 205 days. In these analyses, we were able to collect 2 samples each of hyperplastic glands and two overt tumors with their surrounding tissues to analyze for gene expression. Ar was expressed as a large 25-28 kDa species in tumor tissue at much higher levels than in the surrounding tissue, but was not over-expressed in precancerous tissue, in contrast to Ar expression in PyMT mice (Fig. 2 and Table 1). Another isoform of 14-16 kDa was expressed in normal and tumorous tissues and this increased with age (Fig. 2).

Cr-1, on the other hand, was strongly expressed in 20-week virgin female mammary glands from MMTV-ErbB2 mice that had hyperplastic growths (Fig. 2, lane 2) whereas earlier stages were negative. In the overt tumors in this mouse, however, little if any, was expressed, while Cr-1 was present at low levels in the adjacent tissue. This result is in contrast to the results in MMTV-PyMT mice in which Cr-1 was expressed at greater levels in the tumor itself than in the surrounding tissues and less in the hyperplastic, precancerous gland. In the c-ErbB2 mammary tumor model, Cr-1 is a clear marker for hyperplasia of the mammary gland.

As expected, in MMTV-ErbB2 mice, the levels of ErbB2 expressed were much greater in the tumor tissue than in the hyperplastic or surrounding tissue. In addition, EGFR levels were also much greater in the tumors of these mice than in the surrounding or hyperplastic tissue, suggesting that EGFR is co-induced in these transgenic mice.

MT-hTGFα transgenic mice

The hTGFα-expressing virgin mice do not develop tumors until after approximately 8-9 months, and after multiple pregnancies. Several isoforms of Ar were observed in these samples. A doublet of approximately 26 kDa (large, l) was observed as well as a 14 kDa (small, s) doublet. The higher 26 kDa doublet showed greatest levels of expression in the eighth month transgene positive virgin mammary tissue (Fig. 3, lane 1). Lower levels of expression were observed in the hyperplastic and adenocarcinoma tissues as well as in normal transgene negative pregnant mice. No expression of this doublet was observed in the adenoma or normal male samples or in eight and twenty week virgin transgene positive mice (data not shown). Of more interest, the higher

molecular weight band of the 14 kDa doublet showed greatest levels in hyperplastic and pregnant tissues suggesting that this isoform is expressed in proliferative mammary tissue. The lower molecular weight band showed highest expression in virgin, adenocarcinoma and pregnant tissues.

In the hTGFα expressing mice (Fig. 3), Cr-1 levels were high in the samples from breast hyperplastic tissue. This high level was reached only in the mammary glands of normal pregnant mice. Interestingly, only an intermediate level of Cr-1 remained in the adenocarcinoma samples, while little or none was seen in the adenoma. Cr-1 expression was thus correlated with highly proliferative mammary cells.

EGFR expression was high through 8 months of age (Fig. 3, lane 1). Approximately the same levels were found in the pregnant mammary gland tissue. Slightly less EGFR protein was observed in hyperplasias and adenocarcinomas and significantly less was observed in the adenomas (Fig. 3, lane 4). Interestingly, no ErbB2 was observed in adenomas and normal male mammary glands, while moderate levels occurred in virgin and pregnant mice, and this increased 2- to 3- fold in adenocarcinomas (Fig. 3, lane 3).

Discussion

Mammary tumor progression cannot be readily analyzed in humans and therefore, the transgenic mouse offers the most suitable model to study putative tumor marker expression preceding and during the formation of tumors. Tissue from a single line of mice allows analysis of tumor stages that are fairly consistent in the time of progression to frank tumor formation. We selected Cr-1, Ar, EGFR and ErbB2 to study because of their close association with mammary tumors,

and because these genes are also expressed in normal mammary gland development. Each ligand of the EGFR can up-regulate both the receptor and the other ligands thus increasing the probability of progressive loss of growth control (17). The PyMT-expressing mice develop hyperplasias and tumors the most rapidly of the three models examined, putting these transgenic mice at one extreme of the tumorigenic process. However, the PyMT is a viral gene that is not expressed in the human breast, whereas TGFα and ErbB2 genes are normal gene products overexpressed in human tumor cells, and therefore, these transgenic mouse models are more likely to be closer to the human disease.

We report here that Cr-1 is an early marker for pretumorous/hyperplastic tissue in all 3 transgenic models examined. Cr-1 is known as a proliferative factor for the mammary gland but does not itself appear to cause tumors in mammary cells (4). Cr-1 is upregulated during hyperplastic growth in pretumorous mammary glands, as well as in normal pregnancy. It remains to be determined whether Cr-1 is a suitable marker for early precancerous breast hyperplasia in human patients.

Amphiregulin is a bifunctional polypeptide growth regulator that has been demonstrated to bind solely to the EGFR and induce *in vitro* tyrosine phosphorylation of this receptor in human mammary and ovarian cell lines (18). Ar is already known as an autocrine growth factor in the mammary gland and does appear to cause hyperplastic growth in normal mammary glands (15) but these do not develop further into overt tumors. While Ar becomes overexpressed in hyperplastic MMTV-PyMT mammary tissue and hyperplastic MT-TGFα mammary tissue, we found that the overexpression of ErbB2 stimulates Ar overexpression only at the overt tumor

stage. It has been shown previously that a normal human mammary cell line overexpressing c-ErbB2 (MCF-10A neu cells) displays a 15 to 30-fold increase in the *in vitro* levels of Ar mRNA and protein (16) compared to parental cells. c-ErbB2 is a marker for some tumors and its overexpression is correlated with a poor prognosis. Based on this evidence, ErbB2 expression may be regarded as a late stage marker in the progression to malignant disease.

We were able to show that Cripto is a proliferative factor overexpressed during hyperplastic growth in all mouse mammary tumor models studied. Amphiregulin was overexpressed in 2 of 3 models of hyperplasia. For both genes, the mammary gland in pregnant mice achieved the same high levels of expression, but this fact is unlikely to interfere with the utility of Ar and/or Cr-1 as a marker(s) of the hyperplastic mammary gland.

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FIGURE Legends

Figure 1. Expression analysis of proteins in mammary gland tissues and tumors derived from MMTV-PyMT transgenic mice by immunoblotting. No palpable tumors were present in these tissues. Note that the sample 44+ was hyperplastic with small non-palpable tumors. The (+) or (-) refers to the presence or absence of the transgene. In some analyses α-actinin was used as a loading control, in others, β-actin served this function.

Figure 2. A similar immunoblot analysis of mammary tissues from MMTV-ErbB2 transgenic mice. Four stages are shown and compared to the levels of protein expressed in male mammary gland (lanes 5). All the samples came from transgene positive mice.

Figure 3. A similar immunoblot analysis of the mammary tissues derived from MT-TGFα transgenic mice. Malignant adenocarcinoma tissue is shown in lanes 3 and benign adenoma tissue in lanes 4.

Table 1 Summary of protein expression levels in mammary glands and tumors of transgenic mice

MMTV-PymidT	20d virgin	prehyp	hyperplasia	tumor	pregnant	lactating
Ar	+	+	**++**(l)	+++(l)	++++(l)	+++
Cr	--	-/+	**++**	++++	++++	+++
EGFR	+++	+++	+++	++++	+++	+++
ErbB2	++	+++	+++	++++	+++	+++

MMTV-Neu/ErbB2	prehyp	hyperplasia	tumor	male
Ar	±	±(s)	++++(l)	+(s)
Cr	+/-	**++++**	-	+
EGFR	++	++	++++	+++
ErbB2	++	++	++++	+

MT-TGFα	20 wk	8 mo prehyp	hyperplasia	adenoCa	adenoma	pregnant
Ar	±	++++(l)	**++++**(s)	++	+	++++(s)
Cr	±	+/-	**++++**	++	-	++++
EGFR	++++	++++	+++	+++	++	++++
ErbB2	+++	+++	+++	++++	+/-	+++

Legend At least 2 separate tissue samples were analyzed and the results averaged. ++++ 75-100% (highest level of expression of given protein per tumor system); +++ 50-75%; ++ 25-50%; + 10-25%; -/+ 1-10%; - background. (l) = large; (s) = small isoforms. All isoforms of the proteins were included in the quantification analysis.

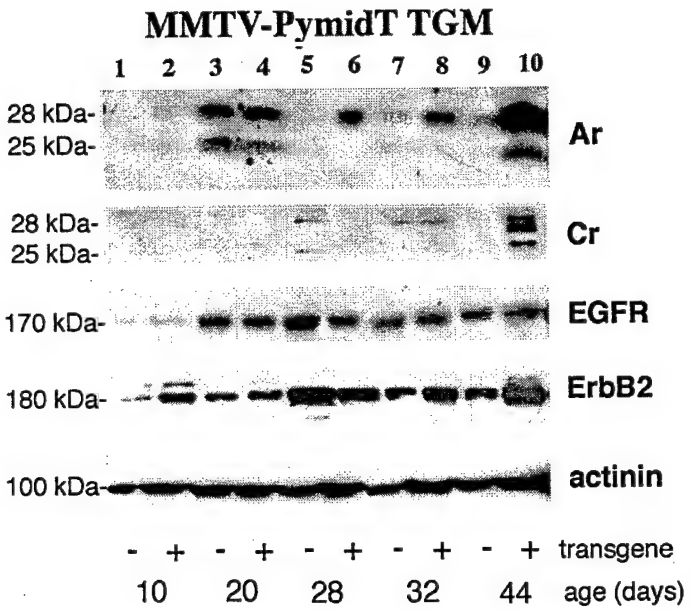

Fig 1

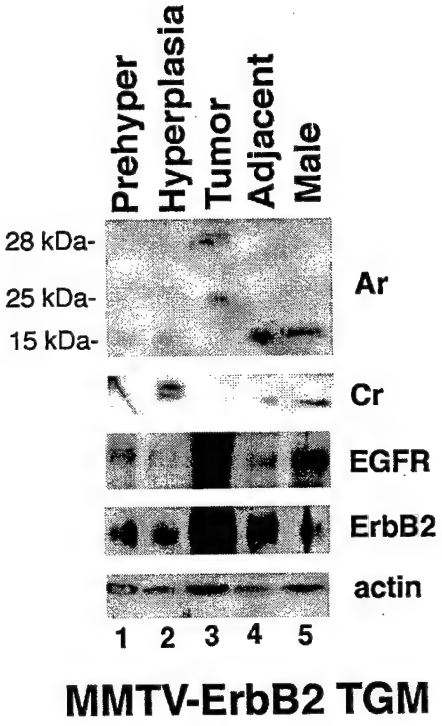

Fig 2

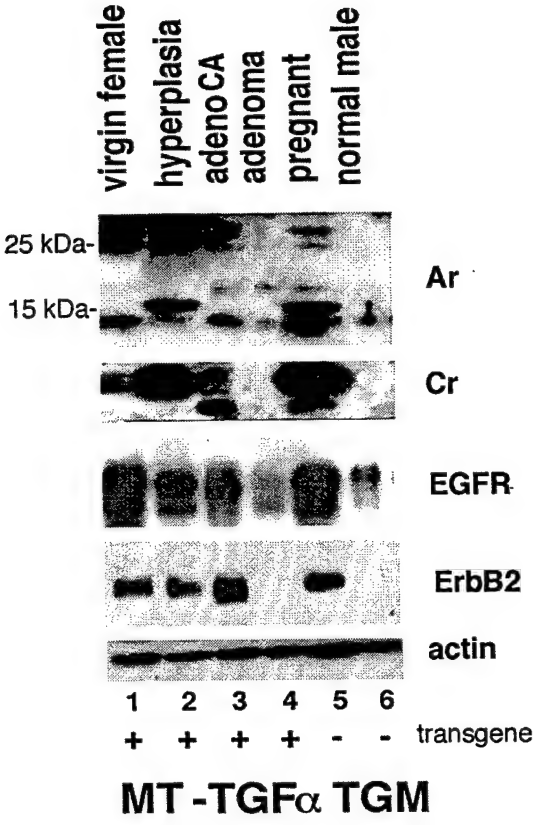

Fig 3

P.N.A.S. June 5, 1998 2 Figs, 3 Tables

Cardiac myosin genes fail to express in developmentally lethal Cripto-1 knockout mice

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Running title: Cripto knockout mice

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ABSTRACT **Cripto-1(Cr-1) protein encoded by the gene (*tdgf-1*), is a secreted growth factor that is expressed early in embryonic development in inner cell mass, primitive streak, and later restricted to the developing heart. The role of Cr-1 was investigated during mouse development by gene targeting. No homozygous Cr-1(-/-) mice were born indicating that Cr-1 is necessary for embryonic development. Embryos initiated gastrulation and produced mesoderm up to day E7.5, but while no normal head-fold or pre-cardiac structures were seen, plentiful mis-folded neuroepithelium was present. Although several biochemical markers of differentiating ectoderm, mesoderm and endoderm were expressed in the Cr-1(-/-) embryos, they lacked all cardiac-specific myosin heavy and light chain expression at E8.7. αMHC, βMHC, MLC2A and MLC2V mRNAs, as well as the cardiac specific ANF were undetectable. Cr-1 protein was shown to act in a paracrine manner to effect rescue of the phenotype of Cr(-/-) cells in chimeric mice. Thus, Cr-1 acts upstream of cardiac myosin genes, and is a master programmer of cardiac organogenesis.**

The mouse Cripto-1 (Cr-1) gene, also known as teratocarcinoma-derived growth factor-1 (*tdgf-1*), encodes a growth factor containing a divergent epidermal growth factor (EGF)-like domain(1). Two of the three EGF bicysteine loops are truncated in the Cr-1protein, and it fails to bind to the EGF receptor(2) or other type I receptor tyrosine kinases in the ErbB family(3). Rather, it interacts with an unidentified receptor and activates intracellular components through ras/raf/MAPK pathway(3). The Cr-1 gene is a member of the CFC gene family identified in human, mouse, frog and zebrafish(4-6), where early developmental roles have been indicated. However, the functions of the Cr-1 gene remains unknown.

The first detected expression of Cr-1 occurs in trophoblast and inner cell mass cells of 4-day mouse blastocysts(7). At E6.5, the primitive streak stage, Cr-1 is expressed in epiblast cells and in the forming mesoderm, but later is restricted to the myocardium of the developing heart tube at E8(8). By E9.5-10 it is expressed strongest in the heart outflow region, conotruncus, when the heart develops into a functional chambered organ(7). After 10.5 days of development, no *in situ* hybridization signals were detected. This highly restricted pattern of Cr-1 expression suggests a role in the important process of gastrulation and the subsequent events leading to heart formation. In support of this, we have shown that disruption of both Cr-1 alleles by homologous recombination in ES cells leads to a specific block in the differentiation *in vitro*, of cardiac myocytes, which suggests that this growth factor plays a role in the specification of cardiac tissues(9).

MATERIALS AND METHODS

Targeting vectors and homologous recombination. The targeting vector used to mutate the Cr-1 gene was constructed and used as described(9). The embryonic stem (ES) cells (R1(10)),were maintained in the undifferentiated state according to the standard protocols(11). The second targeting vector, constructed by insertion of a *hyg* gene beside the *neo* gene, used similarly and hygromycin resistant clones (400 μg/ml) were selected and tested. Screening of targeted clones was done by PCR and Southern blotting(9).

Generation of chimeric mice carrying the disrupted Cr-1 allele. Two targeted ES clones were expanded and used to generate chimeric mice. To obtain chimeras, 10-15 ES cells were microinjected into blastocysts isolated at 3.5 days postcoitum from C57BL/6 pregnant female mice, and then implanted into the uteri of CD-1 pseudopregnant recipient mice(12). The male chimeras, identified by agouti coat color, were bred with Black Swiss and 129SvJ female animals to test for germ-line transmission. Offspring with agouti coat color were tested for the presence of the targeted allele by PCR analysis. Animals that

tested positive for the targeted allele were heterozygous, and these were bred with heterozygous littermates to generate homozygotes.

For the generation of chimeric animals consisting of Cr-1(-/-) ES cells and wild-type Cr(+/+) cell, two clones of ES cells were karyotypically normal and one was injected into 80-100 C57BL/6 blastocysts which were then transplanted into pseudopregnant foster mothers. Tissues from either E14.5 embryos or from 10-day-old mice were dissected and homogenized for assay for their distribution of glucose phosphate isomerase (GPI) isoenzymes as described(12). The Cr-1(-/-) contribution was determined by comparison of the intensity of GPI-1a from the 129SvJ ES cells and the GPI-1b from the C57BL/6 blastocyst, analyzed by the NIH image software.

Genotyping of mice and embryos. Genotype of mice were determined by PCR analysis of genomic DNA from tail biopsies. For embryos, yolk sac or whole embryos were used. Tail, yolk sac or embryos were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 0.1% SDS and 1 mg/ml proteinase K) and sequentially incubated at 55^{0}C for 2-16 hours and 94^{0}C for 10 minutes. The lysates were then subjected to PCR analysis as described(9).

Embryo cultures. Embryos (E7.7 and E8.5) were dissected from decidual extraembryonic tissue was removed, and the embryo transferred to the ES differentiation medium containing 20% FCS in 48-well plates. The cultures were allowed to proliferate for 5 days to 2 weeks, examined for differentiated tissues and beating cardiomyocytes, and collected for genotyping.

Histological and immunohistochemical analyses. Embryos were dissected out of the deciduum and fixed in Z-FIX (Anatech Ltd, Battle Creek, MI) or Sainte-Marie

fixative(13) for 2 hours, transferred to PBS, dehydrated, embedded in paraffin and sectioned at 5 μm onto glass slides. Slides were processed by standard procedures and stained with hematoxylin and eosin. Some sections were immunoperoxidase stained using rabbit serum against AFP or laminin (13, 14).

Immunostaining of embryo outgrowth cultures was performed similarly after culture with a monoclonal antibody, MF20, against myosin heavy chain (MHC), or a monoclonal antibody against 165 kDa neurofilament (2H3, Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD).

RT-PCR Analysis. PolyA$^+$ mRNA was extracted from pooled E8.7 embryos and the cDNA was amplified by PCR using primers and conditions described earlier(9). The sizes of each of the amplified fragments is indicated in Fig.1C. Brachyury (T); GATA4; Mef2C(15); cardiac αmyosin heavy chain (αMHC) and β myosin heavy chain (βMHC)(16); cardiac myosin light chain 2V (MLC2V)(17); cardiac myosin light chain 2A (MLC2A)(18); ANF(9); desmin specific primers, 5'- TGATGAGGCAGATGAGGGAG-3' and 5'-TGAGAGCTGAGAAGGTCTGG-3'; myogenin(9); smooth muscle myosin heavy chain (SMHC) specific primers, 5'-TCCATTCTGTGCACAGGTGAG-3' and 5'-AACTTGCCAAAGCGAGAGGTG-3'. A set of primers for GAPDH(19) was also used as controls. Amplified fragments were separated on a 2.0% agarose gel and visualized by staining with ethidium bromide.

RESULTS

The function(s) of the Cr-1 during development was investigated by generating Cr-1 knockout mice, using the targeting vector, pCR-KO1, previously used for *in vitro* studies (see Fig. 1 in ref(9)), by positive/negative selection(11) in which exons 3, 4 and 5 were replaced by *neo*^{r}. ES R1 cells were electroporated with the linearized targeting vector pCR-

KO1 and ten clones were selected with G418 and ganciclovir and then expanded. To screen for the homologous recombination event, the DNA samples from G418-resistant ES colonies were subjected to PCR amplification using the primers described earlier(9). The primer pairs amplified a 4.2 kb product in ten single knockout (+/-) clones but not in wild-type (+/+) cells(9) genomic DNA gave an expected 4.8-kb fragment. Cr-1 gene targeting was further confirmed by Southern blot analysis (data not shown).

Two independent ES Cr-1 (+/-) cell lines were microinjected into C57BL/6 blastocysts, using standard procedures, to generate 23 male chimeric mice. Most of these gave germ line transmission. Genotypes of mice were determined by PCR analysis of DNA extracted from tail samples (Fig. 1a). Adult heterozygous mice were apparently normal and fertile. Of the 184 progeny derived from heterozygote intercrosses in two different strains, outbred BlackSwiss and inbred 129SvJ, none were homozygous knockouts, indicating that these mice died during gestation and that Cr-1 is essential for embryonic development (Table 1a).

The time of the embryonic lethality was determined by examining genotyped embryos at various stages (Fig. 1b and Table 1b). Most of the live homozygous embryos identified at E6.5 were morphologically normal as judged under the dissecting microscope (data not shown). Therefore, Cr-1(-/-) homozygous embryos implanted and appeared to initiate gastrulation normally. Most of the E7.5-7.7 Cr-1 (-/-) embryos displayed abnormal morphology but developmental arrest occurred mainly at E8-9. The embryonic region of the mutant embryos was dented, distorted in shape, and small compared with normal littermates. At E8.5, all mutants in the Swiss and 129Sv background were markedly smaller than wt (Fig. 2a), with severely distorted embryonic regions (Fig. 2b, c). Mutant Black Swiss and 129SvJ embryos at E9.5 to E10.5, consisted primarily of extraembryonic tissue including yolk sac and ectoplacental cone, while the embryonic region had

degenerated. Mutant visceral yolk sacs (VYS) continued to enlarge from E8.5 to 9.5, but the prominent blood vessels usually present in the normal VYS were absent (compare Fig. 2 d with e). Mice derived from two independent ES cell lines and in both strains of mice examined showed similar malformations.

Histological analysis of paraffin-embedded mutant E8.5 embryos, stained with hematoxylin and eosin, contained the products of all three germ layers (Fig. 2g, h) and extraembryonic structures including the visceral and parietal endoderm, trophoblastic giant cells, amnion and chorion. However, mutant embryos had a shortened axis, and the embryonic region was abnormally inflected producing a distorted neuroepithelial layer (Fig 2i). In some cases, the neuroepithelium formed extra folds that filled the smaller cavity. The presence of early mesoderm was confirmed by detection of the mesoderm marker, brachyury (T), by RT-PCR, in both (-/-) and (+/+) embryos (Fig. 1c). The presence of brachyury is consistent with the expression of this early mesodermal marker in embryoid bodies derived from Cr-1 (-/-) ES cells(9).

Two approaches were used to detect defective tissue development. One was to culture embryos *in vitro* so that any cardiomyocytes in the embryos would have time to form rhythmically contracting masses of cells that could be identified further as cardiac myocytes. Even after extended culture no beating aggregates of cells were seen in mutant embryo cultures, whereas, beating was prominent in wt embryo cultures. Table 2 summarizes the numbers of embryos that were observed and the numbers of embryos that were defective in cardiogenesis. These results were confirmed by staining fixed cultures with an antibody to myosin heavy chain (MF20) (wt, Fig 2 i). No staining was observed in mutant cultures (Fig. 2, j). When the cultures were disaggregated and replated, cultures formed into numerous aggregates of rounded cells that extended axon-like structures. These stained with antibody to neurofilament protein (2H3), confirming that both wildtype

and mutant embryos differentiated to neurons (Table 2). We also showed by immunostaining sections that the visceral endoderm expressed alphafetoprotein (data not shown) and the parietal endoderm and other cells expressed laminin in both wt and mutant embryos (Fig.2, k,l).

The second assay assessed the expression of cardiovascular-specific mRNAs that are usually present at specific stages of development. Individual embryos on day E8.7 were genotyped and pooled into Cr-1(-/-) and (+/+) types. Semi-quantitative RT-PCR was used to probe for the expression of several tissue-specific markers. The cDNAs were examined in a 10-fold dilution series to ensure that the linear range of amplified cDNA was used, and to compare the levels of mRNA in normal and mutant embryos. Figure 1c shows a compilation of the results. The early expressing transcription factors brachyury (T)(20), GATA4(21, 22), and MEF2C(15) were expressed in both normal and mutant embryos. Myogenin, specific for skeletal muscle differentiation(23), was weak but positive in mutant embryos. However, five cardiac-specific marker genes remained unexpressed in mutant embryos: α and β myosin heavy chains (MHC)(16); myosin light chains 2A(18, 24) and 2V(17); atrial natriuretic factor (ANF) were all absent in Cr-1(-/-) embryos but present in wildtype(9). Desmin, a cytoskeletal protein that is one of the earliest markers of future muscle cells(25), was weakly expressed in mutant embryos, indicating that precursors of muscle cells were present in Cr-1(-/-) embryos (Fig. 1c).

Since large blood vessels were not observed in the visceral yolk sacs of mutant embryos, we also tested for markers of endothelial, smooth muscle and blood cells. The VEGF receptor, Flk-1, was expressed, as was tie-1 (observed in sections of E8.5 embryos and around blood islands in VYS using immunostaining, data not shown). The adhesion molecule PECAM, a marker of endothelial cells was expressed in mutant embryos. Flk-1 is necessary for visceral endothelial development(26) and is detected as one of the earliest

markers of endothelium. PECAM and tie-1 expression follows soon after(27). Markers of hemoglobins were detected by RT-PCR (data not shown) in all embryos at E8.5, including globins βH1 and ε^{y}. The smooth muscle specific myosin heavy chain (SMHC)(28) was also expressed (Fig. 1c). The amounts of RNA present in each of the samples was similar, as denoted by the results of RT-PCR assays on GAPDH gene expression(19). In summary the tissues that were absent in Cr-1 null embryos were cardiac tissues.

When the heart fails to beat during development, the embryo dies and the contribution of specific gene functions in later tissues and organs cannot be determined. In order to test if the Cr-1 null embryo can be rescued by the presence of normal embryo cells, chimeric embryos were contructed as follows. ES cells (129Sv) with one disrupted Cr-1 gene were subjected to a further round of gene targeting using a vector modified to select for targeted Cr-1 by hygromycin resistance. One genotyped Cr(-/-) clone was chosen for microinjection into blastocysts derived from the C57BL/6 mouse. 129Sv and C57BL/6 mouse strains have distinct isoforms of the enzyme glucose phosphoisomerase (GPI) that migrate at different rates during cellulose acetate electrophoresis. The composition of the resulting chimeric tissues can be estimated by densitometry of the stained bands on the agarose overlay containing enzyme substrate. Normal embryos were produced that were analyzed on day E14.5 and at 10-days after birth. The results showed that tissues in mosaic animals all contained approximately 50% contribution from mutant cells (Table 3). Hence, mutant ES cells differentiated into all tissues, and were rescued by the normal cells. Since Cr-1 is a secreted protein, this result indicates that a paracrine influence of the Cr-1 protein on Cr(-/-) cells is sufficient to restore normal function.

The most obvious manifestation of the lack of Cr-1 expression in developing embryos is the lack of all cardiac-specific gene expression. Functional cardiomyocytes fail to form, no rhythmic contractions occur, and embryo development ceases. Although muscle precursor

cells were apparently produced, the lack of cardiac myosin prevented further differentiation of the precardiomyocytes. Based on our results for ES cell differentiation(9) skeletal myosins were not affected. Other developmental defects were visible as small embryo size and abnormal dents and folds in the egg cylinder. These embryonic features indicate at least three roles for Cr-1 during development. One is to support the proliferation of specific cell types, for example, human embryonal carcinoma (EC) cells(29), mammary epithelial cells(30) and mammary tumor cells(31). We also found that F9 EC Cr-1(-/-) cells grow at a rate 60-70% of wt (data not shown). The second role is evident as a defect of cell interaction and signaling in the absence of Cr-1, observed as distorted neuroepithelial layers and reduced mesenchymal tissue. Perhaps Cr-1 forms a gradient across the embryonic field, or acts in a chemotactic fashion to cells with Cr-1 receptors, to shape the tissue layers for morphogenesis. The lack of activation of the myosin genes (Fig. 1c) in the absence of active Cr-1 genes is the third defect, and this result indicates that Cr-1 is a major myosin gene activator. The use of the ES cell system and the *in vitro* cardiac differentiation model will be useful in dissecting the molecular mechanisms underlying the activation of myosin genes by Cr-1(9).

The developmental function of Cr-1has been uncertain and so the question of the position of Cr-1 in the cardiac developmental program is important. Our results show that Cr-1 acts upstream of myosin gene activation, but appears to be independent of the pathways involving the important cardiogenic transcription factors Nkx2.5(32), GATA4(33), and MEF2C(22, 34), summarized in reviews(35-37). These transcription factors were all expressed in Cr(-/-) embryos (Fig. 1c) and in differentiating Cr(-/-) ES cells(9). We have therefore demonstrated that Cr-1 is a master gene in mouse cardiac organogenesis. A further implication is that if Cr-1 can influence receptive cells to switch to a cardiogenesis program, there may be a means to treat patients with deficits of cardiac cells.

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FIGURE LEGENDS

Figure 1. Generation of Cr-1 mutant cell lines. (a) Genotypes of adult mice and embryos determined by PCR analysis of tail DNA. A product at 242 bp indicates wt and 4.2 kb for a disrupted Cr-1 gene(9) (b) Genotypes of embryos from Cr-1 (+/-) mating were determined by PCR analysis(9) of embryo lysates. (c) Semi-quantitation of markers by RT-PCR analysis on wt and mutant E8.7 embryos. Samples of cDNA and three serial 10-fold dilutions were analyzed to compare the levels of gene expression. Genes tested were: brachyury (T); GATA4 and Mef2C cardiac related transcription factors; α– and β–myosin heavy chains; atrium-specific myosin light chains (MLC2A) and ventricular-specific myosin light chains (MLC2V); atrial natriuretic factor (ANF); myogenin, (Myo); smooth muscle heavy chain (SMHC); desmin; platelet endothelial cell adhesion molecule (PECAM); the VEGF receptor, Flk-1; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Only the cardiac specific myosins and ANF genes were not expressed in the Cr-1(-/-) embryos.

Figure 2. Analysis of normal and mutant embryos. Normal whole embryos E8.5 (a) and E9.5 (d) and mutant Cr-1 (-/-) embryos at E8.5 (b, c) and E9.5 (e). There is no embryo in the visceral yolk sac in (e), and note the lack of blood vessels. Unlabeled arrows denote abnormal morphology. Histological analysis of sagittal sections of wt embryos at E8.5 (f) and mutant embryos (g, h), stained with hematoxylin and eosin. Embryo immunostaining with antibody to myosin heavy chain after culture. (i) and (j), E 8.5 embryos were cultured as described in the Methods section, and fixed after 5 days when rhythmic beating was observed only in the wt (i) cultures but not in Cr(-/-) embryo cultures (j). (k) and (l), sections of E8.5 embryos immunostained for laminin (black) and counterstained with nuclear fast red. Bars in a-c, 400 microns; d-e, 600 microns; f-h, 350 microns; i-j, 120 microns; k-l, 100 microns. HF, headfolds, H, heart, VYS, visceral yolk sac, NF, neural folds, NE, neural epithelium; PE, parietal endoderm; M, mesoderm.

Table 1a Genotypes of offspring from heterozygous matings

Genetic background	No. of mice	Genotype* +/+	+/-	-/-
BlackSwiss (outbred)	134	48 (35.8%)	86 (64.2%)	0 (0%)
129SvJ (inbred)	50	20 (40.0%)	30 (60.0%)	0 (0%)
Total	184	68 (37.0%)	116 (63.0%)	0 (0%)

*Genotypes were determined by PCR analysis of tail DNA

Table 1b Genotypes of embryos from heterozygous matings

Stage	No. of embryos	Genotype* +/+	+/-	-/-
E3.5	26	7	9	10
E6.5	12	2	6	4
E7.5-7.7	64	19	8	17 (11M)
E8.5	23	6	11	6 (6M)
E9.5	30	8	13	9 (9M)
E10.5	8	2	5	1 (1M)
E11.5	6	3	3	0
Total	169	47 (28%)	75 (44%)	47 (28%)

*Genotypes were determined by PCR analysis of tissue lysate of blastocystoutgrowths for E3.5, embryos for E6.5-8.5 and yolk sac for E9.5-11.5. M, mutated phenotype. The genetic background was BlackSwiss outbred mice.

Table 2 Formation of beating cardiomyocytes and neurons in embryo culture

Stages	No. of embryos	No. of embryos that form neurons	No. of embryos that form cardiomyocytes
E7.7 +/+	5	ND*	5 (100%)
E7.7 +/-	8	ND	8 (100%)
E7.7 -/-	6	ND	0 (0%)
E8.5 +/+	6	6 (100%)	6 (100%)
E8.5 +/-	9	9 (100%)	9 (100%)
E8.5 -/-	4	4 (100%)	0 (0%)

*not done

Table 3 Chimeric mouse analysis

Tissues*	% Cr-1(-/-) ES contribution E14.5 embryos	10d neonates
yolk sac	45.0 ± 1.8	-
Limb	50.0 ± 1.4	-
blood	48.4 ± 5.0	58.3 ± 15.5
brain	46.3 ± 0.7	55.3 ± 10.1
gut	49.7 ± 0.1	33.7 ± 9.0
heart	48.3 ± 0.6	64.0 ± 23.4
liver	48.6 ± 0.9	48.9 ± 9.8
lung	46.6 ± 1.9	49.3 ± 12.7
muscle	-	62.7 ± 11.7
kidney	-	59.2 ± 14.1
spleen	-	40.8 ± 16.5

*Tissues from chimeric mice constructed from Cr-1 (-/-) ES cells and C57BL/6 blastocysts were analyzed at two stages. Each result represents average ± standard deviation of the percentage of ES cell contribution for tissues from 4 embryos or animals.

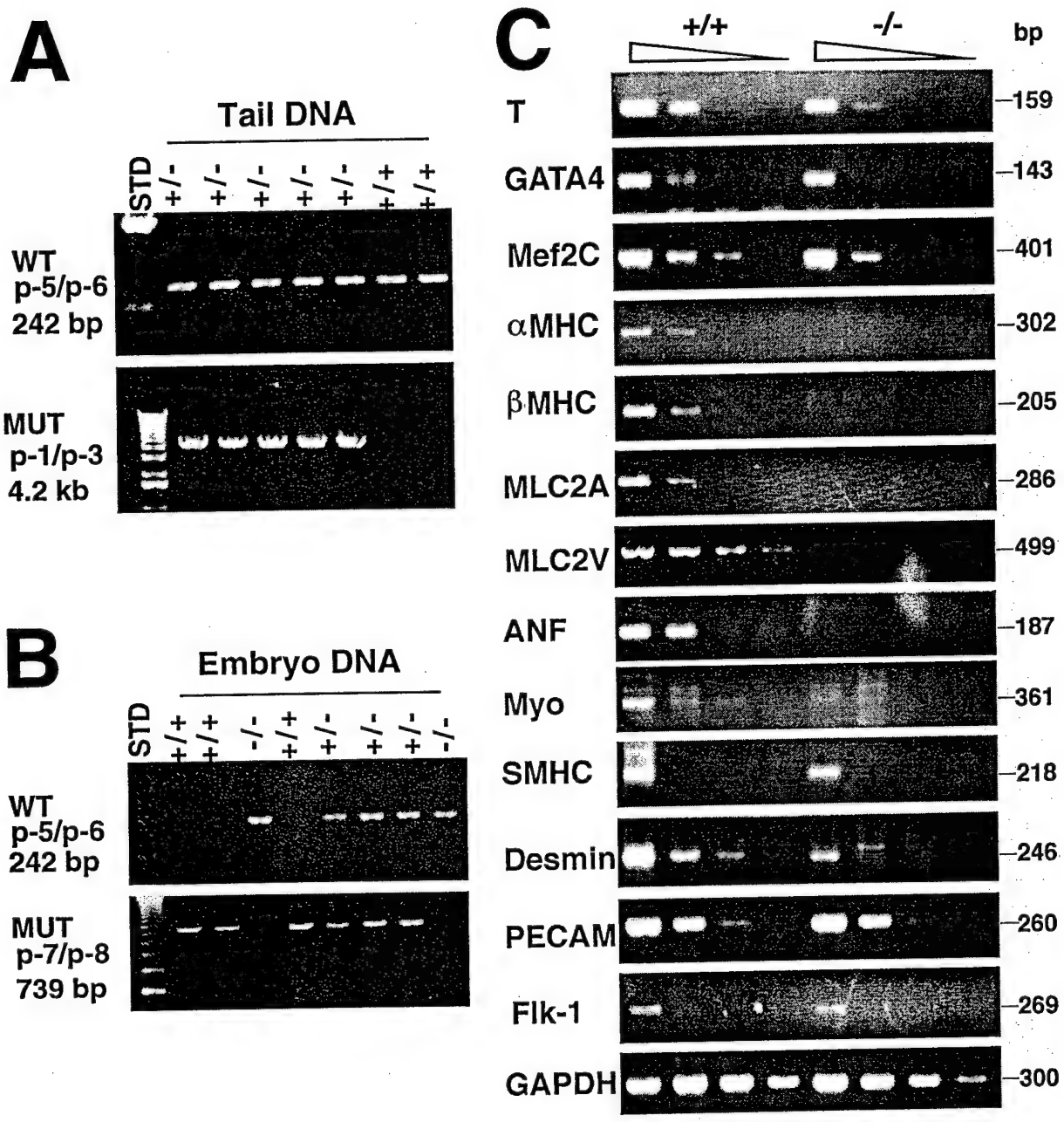
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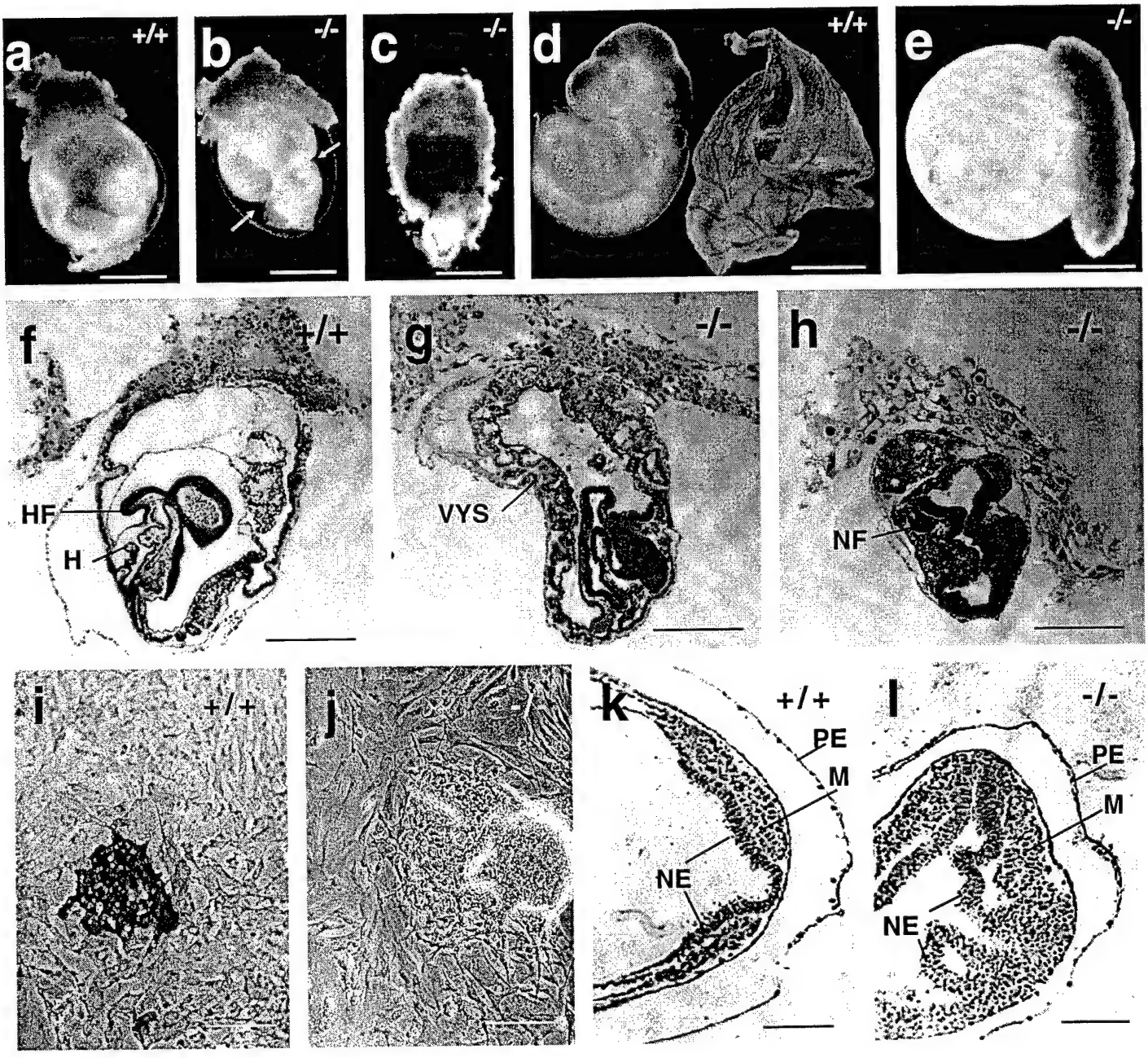
Figure 1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Specific Arrest of Cardiogenesis in Cultured Embryonic Stem Cells Lacking Cripto-1[1]

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The molecular events of cardiac lineage specification and differentiation are largely unknown. Here we describe the involvement of a growth factor with an EGF-like domain, Cripto-1 (Cr-1), in cardiac differentiation. During embryonic development, Cr-1 is expressed in the mouse blastocyst, primitive streak, and later is restricted to the developing heart. To investigate the role of Cr-1, we have generated Cr-1-negative embryonic stem (ES) cell lines by homologous recombination. The resulting double "knockout" ES cells have selectively lost the ability to form beating cardiac myocytes, a process that can be rescued by reintroducing Cr-1 gene back into the Cr(−/−) cells. Furthermore, the lack of functional Cr-1 is correlated with absence of expression of cardiac-specific myosin light and heavy chain genes during differentiation. Differentiation into other cell types including skeletal muscle is not disrupted. These results suggest that Cr-1 is essential for contractile cardiomyocyte formation *in vitro*. © 1998 Academic Press

Key Words: **homologous recombination; gene targeting; markers for ectoderm, mesoderm and endoderm; teratocarcinomas; mutant phenotype; rescue.**

INTRODUCTION

The development of the heart is a complex process composed of two major stages: an early specification and differentiation of cardiomyocytes and later morphogenesis (Olson and Srivastava, 1996). Murine cardiac progenitor cells derive from the anterior lateral plate mesoderm that arises from the primitive streak on the sixth day of gestation (E6.5) (Olson and Srivastava, 1996). These cells differentiate, express cardiac muscle structural genes such as myosin heavy chains and atrial and ventricular myosin light chains (Kubalak *et al.*, 1994; O'Brien *et al.*, 1993), and form the primitive cardiac tube at E7.5–8.0 (Lyons *et al.*, 1990; Morkin, 1993; Sassoon *et al.*, 1988). The newly formed cardiac tube undergoes extensive morphological changes before forming the mature heart. Through gene targeting techniques, many genes have been identified as being involved in the later processes of heart development. However, relatively little is known about the early stage of vertebrate heart development. In this report, we describe the involvement of Cripto-1 (see below) in the early stages of differentiation of cardiomyocytes.

Cripto-1 (Cr-1), encoded by the *teratocarcinoma-derived growth factor-1* (*tdgf*-1) gene (Liguori *et al.*, 1996), is a growth factor with an epidermal growth factor-like motif (Ciccodicola *et al.*, 1989). Although Cr-1 folds in the characteristic EGF-like manner using its six cysteine residues, the resulting polypeptide is unable to bind to members of the EGF receptor family due to truncation of some sequences (Brandt *et al.*, 1994). Cr-1 is a growth factor for mammary cells by interaction with an unidentified receptor to activate intracellular components through ras/raf/MAPK pathway (Kannan *et al.*, 1997). Cr-1 and the related Cryptic gene have been assigned to a distinct family, with the proposed name, CFC (Shen *et al.*, 1997).

Both human and mouse *Cr-1* are encoded by 6 exons (exon 4 contains the EGF-like domain) producing (in mouse) a secreted protein of 171 amino acid residues (Liguori *et al.*, 1996). *Cr-1* is expressed at three main stages in development. The earliest expression of Cr-1 occurs in 4-day mouse blastocysts in both trophoblast and inner cell mass cells (Johnson *et al.*, 1994). At day 6.5, Cr-1 is present in epiblast

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cells and is strongly expressed at the primitive streak stage in the forming mesoderm. Then, a pattern restricted to developing heart structures occurs (Dono *et al.*, 1993; Johnson *et al.*, 1994) with Cr-1 specifically expressed in the myocardium of the developing heart tubes in 8.5-day-old embryos and in the outflow region, conotruncus, of the heart at 9.5–10 days gestation, when the heart develops into a functional chambered organ. After 10.5 days of development, no *in situ* hybridization signals have been detected in the embryo (Dono *et al.*, 1993). This pattern of Cr-1 expression suggests a role in the process of gastrulation, wherein the ectoderm germ layer gives rise to the mesoderm tissues including those producing the heart and somites. In addition, the highly restricted expression patterns of Cr-1 raise the possibility that Cr-1 may play a role in regulating cardiac gene expression. We used the ES[3] model system to test this possibility.

MATERIALS AND METHODS

Construction of targeting vectors. The targeting vectors were constructed with the pPNT plasmid (Tybulewicz *et al.*, 1991). In the vector pCR-KO1, a 5-kb 5′ homologous sequence containing exon 1 and 2 was inserted in front of a gene cassette encoding neomycin phosphotransferase (*neo*) gene under the control of the phosphoglycerokinase (PGK) promoter. A 3.8-kb 3′ homologous fragment containing exon 6 was cloned into the *Bam*HI site downstream to the *neo*. The targeting vector pCR-KO2 was prepared by inserting the PGK–hygromycin (PGK–*hyg*) downstream of the PGK-*neo* in pCR-KO1. Negative selection to avoid random insertion was provided by the herpes simplex virus thymidine kinase (hsv–tk) gene.

Embryonic stem cell culture, electroporation, and selection. The ES cells were maintained in the undifferentiated state by culture on mitomycin C-treated mouse embryonic fibroblasts (MEF) feeder layers according the standard protocols. The medium used was high glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1× nonessential amino acids, 2 mM glutamine, 100 units penicillin/ml, 0.1 mg of streptomycin, and 2% leukemia inhibitory factor (LIF) (conditioned medium collected from confluent cultures of Chinese hamster ovary cells transfected with the LIF expression plasmid provided by permission of the Genetics Institute, Andover, MA). The fetal bovine serum was obtained from Tissue Culture Biologicals (Tulare, CA).

For the generation of Cr-1 (+/−) cells, 1×10^7 R1 ES cells were electroporated with 30 μg linearized pCR-KO1 plasmid DNA at 300 V, 250 μF. Recombination events were selected with G418 (380 μg/ml) and gancyclovir (0.2 μM). To disrupt the second allele, Cr-1 (+/−) E31 and E40 cells were electroporated with linearized vector pCR-KO2 and selected in medium containing hygromycin B (400 μg/ml) and gancyclovir (0.2 μM).

Differentiation of ES cells. Embryoid bodies (EBs) were formed by ES cell aggregate culture in hanging drops (Wobus *et al.*, 1991). The differentiation medium was ES culture medium containing 20% fetal calf serum but without LIF. Rhythmic beating of the EBs, indicating cardiac muscle differentiation, was monitored by daily inspection of the cultures using phase microscopy. Induction of differentiation into skeletal muscle was performed by the method similar to that described by Weitzer (Weitzer *et al.*, 1995). Induction of differentiation of ES cells into neurons was performed by a method similar to that described by Bain (Bain *et al.*, 1995).

Screening for recombinant clones. Genomic DNA samples were extracted from individual clones and examined for the presence of a targeted allele by PCR analysis using *Taq* Extender PCR additive system (Stratagene, San Diego, CA) or Expand PCR System (Boehringer Mannheim, Indianapolis, IN) with following primer pairs. The Cr-1-specific primers, 5′-ACCTGCTCTGTGTCTCCTGATTTCC-3′ (p-1), which is outside (3′) of the targeting vector, and 5′-CACTAGGTGACCAATCTGGTCCAAC-3′ (p-2), a *neo* gene-specific primer, 5′-GCAGCCTCTGTTCCACATACACTTC-3′ (p-3), and a *hyg* gene-specific primer, 5′-TCACGTTGCAAGACCTGCCTGAAAC-3′ (p-4). The PCR conditions were as follows: 94°C for 4 min; 10 cycles of 94°C for 10 s, 65°C for 30 s, and 68°C for 4 min, and followed by 20 cycles of 94°C for 10 s, 65°C for 30 s, and 68°C for 4 min with 20-s increments for each cycle and a final extension step at 72°C for 10 min. Additional PCR reaction using primers within intron 3: 5′-GTCCCTGATAGTCTCTGATATTC-3′ (p-5) and 5′-GAAATGTAAGAAAAGTCATGGGG-3′ (p-6) or a set of primer in *neo* gene: 5′-GTCAAGAAGGCGATAGAAGGCGATGCG-3′ (p-7) and 5′-GGTGGAGAGGCTATTCGGCTATGACTG-3′ (p-8) was also performed. The PCR reaction for primer p-4/p-5 and p-6/p-7 was performed by denaturing the DNA at 94°C for 2 min, followed by 35 cycles of amplification: 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min, and a final extension step at 72°C for 6 min.

RT–PCR Analysis. Total RNA (3 μg) was isolated from ES cells and EBs, treated with RNase-free DNaseI (Stratagene, San Diego, CA) and converted into cDNA using oligo(dT) as a primer. The cDNA was amplified by PCR using primers within the Cr-1 deleted region, 5′-ATCGGTCTTTCCAGTTCGTGCCTTC-3′ in exon 3 and 5′-ATCTGCACAGGGAACACTTCTTGGG-3′ in exon 5. A set of primers for GAPDH (Hummler *et al.*, 1996), 5′-CGTCTTCACCACCATGGAGA-3′ and 5′-CGGCCATCACGCCACAGTTT-3′, was also added to the same reaction as controls. The cDNA from Day 10 EBs was amplified by PCR using specific primers for MLC2v (Miller-Hance *et al.*, 1993); specific primers for ANF, 5′-CGGTGTCCAACACAGATCTG-3′ and 5′-TCTCTCAGAGGTGGGTTGAC-3′, which should give a 187-bp product; specific primers for desmin, 5′-TGATGAGGCAGATGAGGGAG-3′ and 5′-TGAGAGCTGAGAAGGTCTGG-3′, which should give a 246-bp product, and GAPDH (Hummler *et al.*, 1996). The cDNA from Day 6 EBs was amplified by PCR using brachyury-specific primers, 5′-ATCAAGGAAGGCTTTAGCAAATGGG-3′ and 5′-GAACCTCGGATTCACATCGTGAGA-3′, which should give a 159-bp product; GATA4 specific primers, 5′-CACTATGGGCACAGCAGCTCC-3′ and 5′-TTGGAGCTGGCCTGCGATGTC-3′, which should give a 143-bp product; Mef 2C-specific primers (Martin *et al.*, 1993); Nkx2.5-specific primers (Biben and Harvey, 1997), 5′-TGCAGAAGGCAGTGGAGCTGGACAAGCC-3′ and 5′-TTGCACTTGTAGCGACGGTTCTGGAACCAG-3′, which should give a 220-bp product; Egr-1-specific primers, 5′-CCGAGCTCT-

[3] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ES cells, embryonic stem cells; EB, embryoid body; KO, knockout; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts; MHC, myosin heavy chain; MLC, myosin light chains; PGK, phosphoglycerokinase; tk, thymidine kinase; SRF, serum response factor.

TCACACAACAACTTTTGTC-3′ and 5′-CCGAGATCTCCCAG-CTCATCATCAAAC-3′, which should give a 355-bp product and GAPDH primers. cDNA samples from Day 28 EB outgrowths were tested for skeletal muscle differentiation by PCR using myogenin-specific primers selected from sequences in GenBank: 5′-TGA-GGGAGAGCGCAGGCTCAAG-3′ and 5′-TGCTGTCCACGAT-GGACGTAAGG-3′, which should give a 361-bp product. cDNA samples derived from Day 25 EB outgrowths were tested for neuronal differentiation by PCR amplification using light chain neurofilament-specific primers, derived from GenBank: 5′-CTCCTA-CTTGATGTCTGCTCGC-3′ and 5′-TCAGACTCATCCTTGGC-AGC-3′, which should give a 219-bp product.

The PCR reactions for Cr-1, GAPDH, desmin, brachyury, GATA4, MEF2C, Egr-1, myogenin, and neurofilament were performed by denaturing the DNA at 94°C for 2 min, followed by 35 cycles of amplification: 94°C for 30 s, 60–65°C for 30 s, 72°C for 1.5 min, and a final extension step at 72°C for 6 min. The RT–PCR assays for MLC2V were performed by denaturing the DNA at 95°C for 5 min, followed by 35 cycles of amplification: 94°C for 30 s, 70°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 6 min. The RT–PCR assays for ANF were performed by denaturing the DNA at 95°C for 5 min, followed by 35 cycles of amplification: 94°C for 30 s, 55°C for 30 s, 72°C for 50 s, and a final extension step at 72°C for 6 min. The RT–PCR assays for Nkx2.5 were made from cDNA converted by the Nkx2.5-specific 3′ primer and performed by denaturing the DNA at 94°C for 4 min, followed by 35 cycles of amplification: 94°C for 30 s, 59°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min.

Western blotting. ES cells were cultured as aggregates to form EBs that continuously produced many differentiated tissues during the time frame (Keller, 1995). EBs derived from different cell lines during a time course of differentiation were dissolved in lysis buffer (Laemmli, 1970), separated by SDS–PAGE, and then transferred to polyvinyline difluoride membrane (Immobilon, Millipore Corp., Bedford, MA) using standard Western blotting methods (Burnette, 1981). The samples were detected for myosin heavy chain (MHC) by a monoclonal antibody MF20 (Developmental Studies Hybridoma Bank, NICHD, NIH), for MLC2A by a rabbit antiserum (a kind gift from Dr. K. Chien, University of California at San Diego), for ErbB2 and ErbB3 by rabbit antibodies (Santa Cruz Biotechnology Inc., CA), and for AFP and laminin by rabbit antisera (Dziadek and Adamson, 1978; Grover *et al.*, 1983). The blots were then incubated with the peroxidase-labeled secondary antibodies. Signals for binding of the antibodies were detected by enhanced chemiluminescence system (ECL Amersham Corp., Aylesbury, UK). For controls, the blots were reprobed with mouse monoclonal antibodies against β-actin or α-actinin (Sigma, St. Louis, MO) using a method developed by (Krajewski *et al.*, 1996).

Stable transfection for rescue. Cells were cotransfected by lipofectamine (Gibco-BRL, Gaithersburg, MD) and plasmid vectors containing Cr-1 sense or antisense cDNA driven by the SV40 promoter together with a plasmid DNA containing puromycin resistance gene driven by the PGK promoter, according to the manufacturer's recommendation. One week after the selection with 1.6 μg/ml puromycin, resistant clones were pooled, expanded, and subjected to the differentiation assay.

To test for the reexpression of Cripto-1 transcripts in these cultures, semiquantitative RT–PCR was performed. Each cDNA sample was 10-fold serially diluted and used as templates for the PCR amplification with specific primers for the Cr-1 gene, using GAPDH gene transcripts as loading controls. The DNA bands produced after 35 cycles of PCR were matched to compare levels between cell lines.

Teratocarcinoma. Cells (6×10^6) were injected into two subcutaneous sites of 6-week-old 129SvJ mice. Five mice were used for each cell line. The tumors were processed for histological analysis 3 weeks after the injection. The identification of tissue types was made by a board-certified pathologist, Dr. D. Mercola, M.D., Ph.D., FACP.

RESULTS

Targeting the Cripto-1 Gene in ES Cells by Homologous Recombination

To precisely define the function(s) of Cr-1, we have disrupted both copies of the wild-type Cr-1 alleles sequentially by homologous recombination in R1 (Nagy *et al.*, 1993) ES cells. The two targeting vectors, pCR-KO1 and pCR-KO2, caused the replacement of Cr-1 exons 3, 4, and 5 by PGK-*neo* and by PGK-*neo* -*hyg*, respectively, as determined by PCR analysis of genomic DNA (Figs. 1a–1d) and Southern blotting analysis (data not shown). To further confirm the Cr-1 (−/−) mutation, reverse transcriptase–polymerase chain reaction (RT–PCR) was performed to examine whether Cr-1 mRNA was present in the clones isolated. No Cr-1 cDNA could be amplified from homozygous mutant cells while a 216-bp product was obtained from wild-type and Cr-1 (+/−) clones (Fig. 1e). In the same reaction, a 300-bp fragment of GAPDH was produced from all the samples (Fig. 1e). These tests confirmed that the Cr-1 gene was completely inactivated in the R1-derived clones selected.

ES Cell Differentiation as Aggregates in Hanging Drops

Mouse ES cells differentiate *in vitro* into a variety of cell types including spontaneously contracting cardiac myocytes (Keller, 1995). Since Cr-1 is expressed in the developing heart during embryogenesis, we examined whether Cr-1 knockout had any effect on cardiomyocyte formation by *in vitro* differentiation analysis. Comparisons were made among several clones, including wild-type R1, Cr-1 (+/−) E31, and E40, two independent Cr-1 (−/−) ES cell lines, DE7 and DE39, obtained from transfection of Cr-1 (+/−) E31 cells, and two other independent Cr-1 (−/−) lines, DE14 and DE48, derived from Cr-1 (+/−) E40 cells. Differentiating cultures were initiated by removing ES cells from the feeder layer and forming size-controlled aggregates in hanging drop cultures (Wobus *et al.*, 1991). All cell lines tested gave rise to embryoid bodies (EBs) of similar sizes and gross morphology (data not shown). After 7 days in culture, spontaneously contracting EBs were observed in cells derived from wild-type R1 cells. The beating activity was also observed in EBs derived from Cr-1 (+/−) E31 and E40 after 9 days in culture. The duration of continuous spontaneous contractile activity in cardiac myocytes from R1, E31, or E40 was 1 to 2 weeks. In contrast, no beating cardiac cells were observed in EBs derived from any of the four independent Cr-1 (−/−) ES clones tested (Table 1), even during culture for an

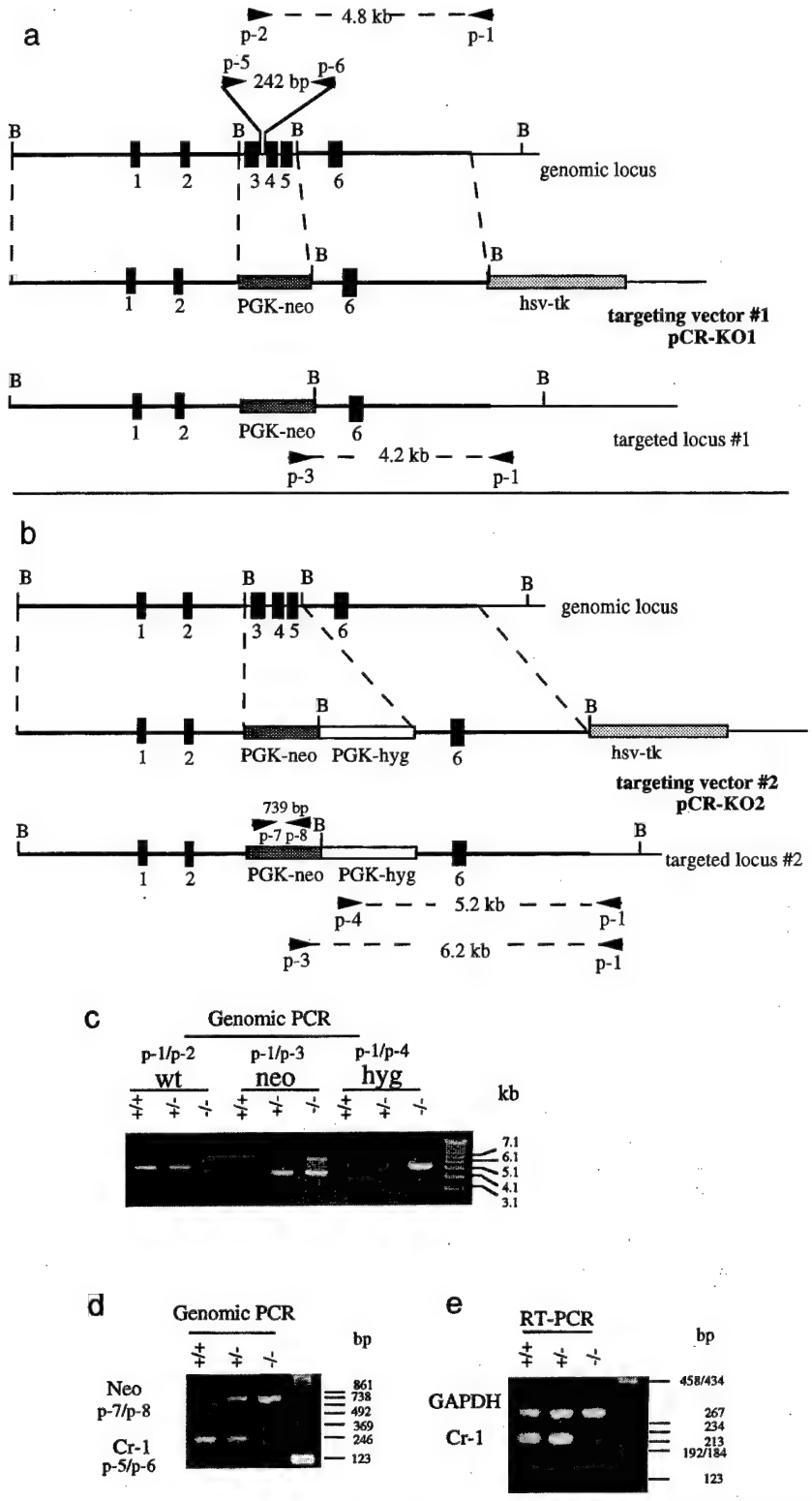

FIG. 1. Generation of Cr-1 (−/−) cells. (a and b) Strategies for disrupting Cr-1 alleles. Exons are depicted by the open boxes and numbered. B represents *Bam*HI. (c and d) Genotyping of cell lines. The primer p-3 and p-1 amplified a 4.2-kb product in Cr-1 (+/−) clones and 4.2-kb and 6.8-kb products in Cr-1 (−/−) cells (under neo). The p-1 and p-2 amplified a 4.8-kb product from Cr-1 (+/+) and (+/−) cells (under wt). The p-4 and p-1 gave a 5.2-kb product only in the Cr-1 (−/−) cells (under hyg). The p-5 and p-6 amplified a 242-bp product from Cr-1 (+/+) or (+/−) cells while the p-7 and p-8 amplified a 739-bp product from Cr-1 (+/−) and (−/−) cells. (e) RT–PCR verification of Cr-1 mutations.

TABLE 1
Inhibition of Cardiomyocyte Differentiation in Cr-1 (−/−) Cells

Cells	No. of EBs	Percentage of EBs with beating cardiomyocyte								
		D6	D7	D8	D9	D10	D11	D12	D13	D14
R1 (+/+)	55	0	32.7	52.7	58.2	38.2	32.7	14.5	9.0	7.2
E31 (+/−)	46	0	0	0	4.3	10.9	8.7	2.2	2.2	2.2
E40 (+/−)	49	0	0	0	18.4	30.6	34.7	24.5	24.5	16.3
DE29 (+/−)	37	0	0	0	5.4	13.5	10.8	10.8	8.1	5.4
DE32 (+/−)	48	0	0	14.5	20.8	29.2	14.6	12.5	12.5	10.4
DE7 (−/−)	52	0	0	0	0	0	0	0	0	0
DE14 (−/−)	43	0	0	0	0	0	0	0	0	0
DE39 (−/−)	46	0	0	0	0	0	0	0	0	0
DE48 (−/−)	34	0	0	0	0	0	0	0	0	0
DE7S	41	0	0	0	2.4	4.9	7.3	7.3	4.9	2.4
DE7A	45	0	0	0	0	0	0	0	0	0

Note. EBs were cultured from wild-type ES cells, R1(+/+), from cell lines with one Cr-1 allele mutated, E31 and E40 (+/−), from cell lines with both Cr-1 alleles mutated, DE7 (−/−) and DE39 (−/−) (derived from E31), DE14 (−/−), and DE48 (−/−) (derived from E40). Cells that remained heterozygous after the hygromycin selection, DE29 (+/−) and DE 32 (+/−), were also tested for the ability and timing of beating cardiomyocyte formation. DE7(−/−) cells transfected with a plasmid containing Cr-1 sense, population DE7S, or antisense Cr-1 cDNA, population DE7A, were monitored for beating cardiomyocyte aggregates from Days 6 to 14. The rescue of beating in Cr-1(−/−) cells occurred only in the sense-expressing cultures. Similar results were found in more than three independent experiments. D, day.

additional 10 days (data not shown). Furthermore, in addition to the difference in the time for the first appearance of beating, the wild-type R1 cells gave rise to a higher percentage of beating EBs than Cr-1 (+/−) cell lines, indicating this effect was dose-dependent (Table 1). These results indicate that the formation of cardiac myocytes was completely blocked by Cr-1 mutations in ES cells. Inactivation of Cr-1 did not appear to influence cell growth of undifferentiated mouse ES cells as measured by proliferation assays (data not shown), although growth inhibition was reported for a human teratocarcinoma cell line treated with an antisense CR-1 vector (Baldassarre *et al.*, 1996).

The observed phenotype was not due to clonal variation since four independent Cr-1 (−/−) clones derived from transfection of two Cr-1 (+/−) cell lines displayed the same phenotype while none of the Cr-1 (+/+) or (+/−) cell lines was defective in cardiac myocyte formation. Nor was the phenotype a result of the drug selection, since two clones, DE29 and DE32, that remained heterozygous after the hygromycin selection formed beating EBs with similar percentages to Cr-1 (+/−) cell lines (Table 1).

Rescue of the Mutant Phenotype

To definitively confirm that the block to cardiac differentiation was due to the inactivation of Cr-1, we cotransfected one Cr-1 (−/−) cell line DE7 with a full-length Cr-1 sense or antisense expression plasmid together with a plasmid for puromycin resistance. Stable clones were selected for resistance to puromycin, pooled as a population of cells, and examined for cardiac differentiation. The resulting population, DE7S, was found to have regained the ability to differentiate into beating cardiomyocytes while a population of cells selected after transfection with antisense Cr-1, DE7A, were still unable to differentiate (Table 1). The percentage of EBs that resumed beating was lower than in wt ES cells because in cotransfections not all the cells in the puromycin-resistant population take up and express the Cr-1 gene. The expression of Cr-1 mRNA in cultures that were "rescued" for the beating phenotype was confirmed by the detection of Cr-1 mRNA only in rescued DE7S-(−/−R) cells, in Cripto-1 (+/−) (Fig. 2) and in wt ES cultures (Fig. 1). Semiquantitative analysis of the relative expression of Cr-1 in rescued clones showed that about 10-fold less Cr-1 was expressed in the rescued population than in the heterozygous (+/−) cells (Fig. 2). In accord with these levels of Cr-1 expression, the frequency of beating reached 7% in rescued cells versus 35% in heterozygous cells. Therefore,

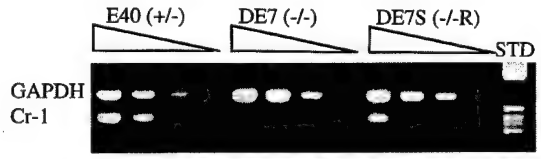

FIG. 2. Semiquantitative RT–PCR analysis of Cr-1 RNA expression in Cr-1 (+/−) and rescued Cr-1 (−/−R) cells. Cr-1 expression was estimated using 10-fold serial dilutions of cDNA that were subjected to the PCR analysis using two sets of primers designed to detect Cr-1 and GAPDH. RNA isolated from Cr-1 (+/−) E40 cells, Cr-1 (−/−) DE7 cells, and rescued Cr-1 (−/−R) DE7S cells were compared by reference to the signal observed as ethidium bromide-stained amplified DNA in each dilution series.

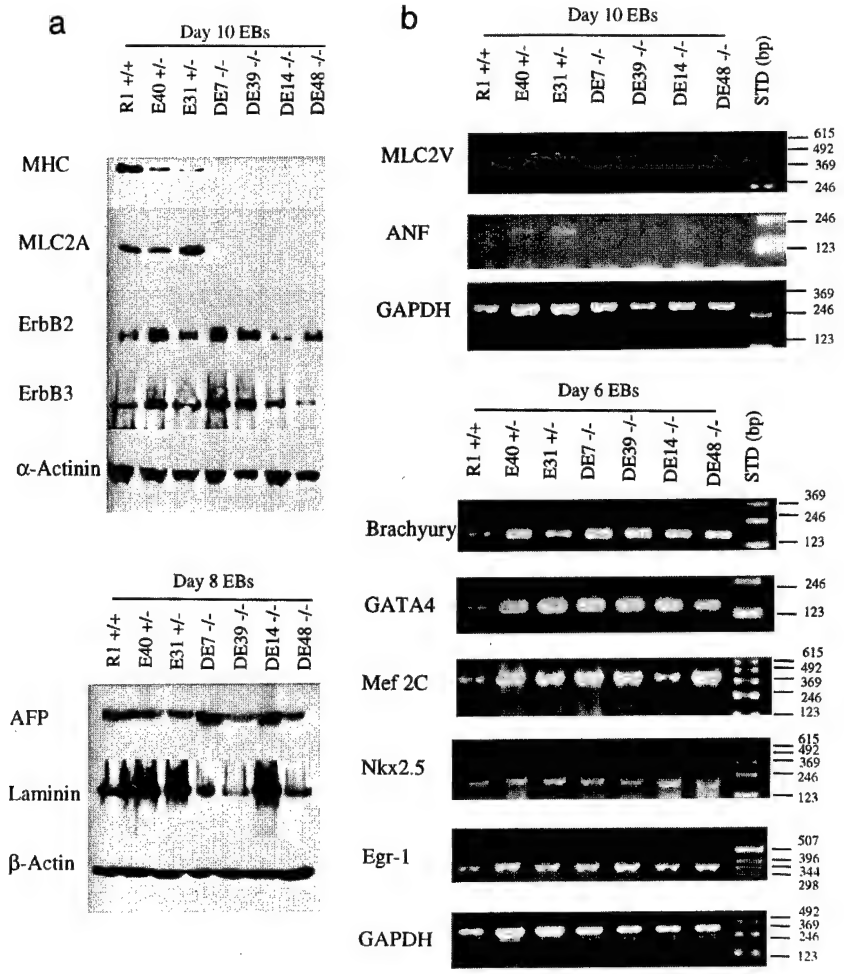

FIG. 3. (a) Western blot analysis of EBs. EBs derived from indicated cell lines at the specific day were detected for myosin heavy chain (MHC) by a monoclonal antibody MF20 (Developmental Studies Hybridoma Bank, NICHD, NIH), for MLC2A by a rabbit antiserum (a kind gift from Dr. K. Chien, Dept. of Medicine, University of California at San Diego), for ErbB2 and ErbB3 by rabbit antibodies (Santa Cruz Biotechnology Inc. CA), and for AFP and laminin by rabbit antisera. For a control, the blot was reprobed with antibody against β-actin or α-actinin. Signals for binding of the antibodies were detected by the enhanced chemiluminescence system (b) RT–PCR detection of MLC 2V, ANF, and GAPDH in Day 10 EBs; brachyury, GATA4, Mef2C, Nkx2.5, Egr-1, and GAPDH in Day 6 EBs.

differentiation to cardiomyocytes is specifically restored by Cr-1 while differentiation is specifically blocked by the inactivation of the Cr-1 gene.

Assays for Markers of Cardiac Differentiation

To determine where Cr-1 might be acting during the process of cardiac differentiation, we examined the expression of the myosin heavy chain (MHC) and the cardiac-specific myosin light chain 2A (MLC2A), two major contractile proteins of cardiomyocytes, in 10-day old EBs from each cell type by Western blotting. As shown in Fig. 3a, the strongest signal for MHC was detected in EBs derived from wild-type R1 cells. Cr-1 (+/−)-derived EBs gave weaker signals compared to that of wild-type EBs. In contrast, expression of MHC in Cr-1 (−/−) EBs was undetectable. Similarly, expression of MLC2A was observed only in the wild-type and Cr-1 (+/−)-derived EBs but not in Cr-1 (−/−) EBs. Transcripts of cardiac-specific myosin light chain 2V (MLC2V) were undetectable in Cr-1 (−/−) EBs as examined by RT–PCR (Fig. 3b, line 1). The expression of myosin polypeptides precedes contractile activity and so components upstream of the myosin induction pathway must be affected. An early marker of muscle cell differentiation, desmin, the intermediate filament protein, was present as transcripts in both wild-type and mutant cell cultures (data not shown), while transcripts of a later marker of heart differentiation, atrial natriuretic factor (ANF) were absent only in the mutant 10 day EB cultures (Fig. 3b, line 2).

As detected by RT–PCR, transcripts of precardiac mesoderm markers, transcriptional factors GATA4 (Edmondson *et al.*, 1994; Heikinheimo *et al.*, 1994), were present in Cr-1 (−/−) cell culture (Fig. 3b). The homeobox gene, Nkx2.5, is related to *tinman* in *Drosophila*, where it is known to be essential for heart formation and has been implicated in the early stages of mouse cardiac myogenesis and morphogenesis (Lints *et al.*, 1993) in mice produced using knockout technology (Biben and Harvey, 1997; Lyons *et al.*, 1995). To determine if Cr-1 acted upstream of the expression of this gene, we analyzed differentiating EBs for Nkx2.5 mRNA by RT–PCR. As shown in Fig. 3b, transcripts of Nkx2.5 were detectable in Cr-1 (−/−) EBs. Egr-1, another transcription factor, which has been shown to be involved in stimulation of cardiac MHC expression (Gupta *et al.*, 1991), is expressed and is not affected by inactivation of Cr-1 (Fig. 3b). Expression of ErbB2 and ErbB3 in Cr-1 mutant EBs was not significantly different from wild-type EBs (Fig. 3a). This family of genes are involved in later stages of cardiac development because inactivation of ErbB2, ErbB3, ErbB4, and heregulin in mice leads to inhibition of cardiac muscle trabeculae formation and causes midgestational death (Erickson *et al.*, 1997; Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995).

The muscle-specific actin genes occur in four isoforms in cardiac, skeletal, enteric, and vascular muscle cells. Their ontogenesis has been described in ES cells and cardiac α-actin; transcripts appear on Day 5 of differentiation (Ng *et al.*, 1997). We used the same experimental approach to assay for the appearance of cardiac α-actin transcripts in ES and differentiated EBs. In contrast to Ng *et al.*, we found that even undifferentiated ES cells express transcripts for α-cardiac actin and continue to do so throughout the time course of differentiation. This was tested using the same conditions for RT–PCR as described by Ng *et al.*, as well as using different primers under more stringent conditions (data not shown). The same positive result was obtained for both Cr-1 (−/−) and Cr(+/+) genotypes, indicating that this gene is poorly regulated in ES cells, and is not a good marker for cardiac specific differentiation.

Assays for Markers of Other Differentiated Tissues

To test the possibility that Cr-1 disrupts early stages of mesoderm differentiation, we examined the expression of an early mesoderm marker, brachyury (T) (Wilkinson *et al.*, 1990), since cardiomyocytes are mesoderm-derived. As shown in Fig. 3b, brachyury transcripts were present in 6-day EBs derived from all cell lines tested, including wild-type R1, two Cr-1 (+/−) cell lines, and four Cr-1 (−/−) cell lines as detected by RT–PCR. Therefore, inactivation of Cr-1 did not affect expression of this early mesoderm marker. We then evaluated the role of Cr-1 on the generation of other mesoderm-derived cell types such as skeletal muscle by culturing EBs on gelatin-coated dishes. Both wild-type and mutant EBs attached to the dishes within 1 day and differentiating cells grew outward. Cardiac contraction started strongly in the dense clusters of attached wild-type R1 aggregates but was never observed in the Cr-1 (−/−) cells as described above, in agreement with the absence of MLC2A in outgrowths of Cr (−/−) EBs (data not shown). Small myotubes started to form in both wild-type and mutant cell cultures after 2 weeks in culture and myotubes continued to grow into large skeletal muscle-like myotubes (Fig. 4a) that contained multiple nuclei and contracted frequently on Days 20–25. The formation of skeletal muscle was further confirmed by expression of a skeletal-specific gene, myogenin, as detected by RT–PCR. Transcripts were detected in RNA extracts of all differentiated ES cell lines tested whether Cr-1 expressing or not (Fig. 5, top line).

Differentiation into other mesoderm and into other cell types was demonstrated by the generation of teratocarcinomas, which form a wide range of recognizable differentiated cell types. To examine differentiation *in vivo*, wild-type R1 cells and Cr-1 (−/−) DE39 cells were injected (sc) into 129 SvJ mice. After 3 weeks, tumors formed from both the mutant cells and wild-type cells and examination of hematoxylin and eosin-stained sections indicated that the teratocarcinomas contained many cell types. Dermis and squamous epidermis (Fig. 4c), cartilage (Fig. 4d), and epithelioid organs (Fig. 4e) were observed in teratocarcinomas derived from both cell lines. Therefore, inactivation of Cr-1 did not affect differentiation of other mesoderm cell types in spite of the inability to differentiate into cardiomyocytes.

To test if the mutant cells can differentiate into neurons, an ectoderm-derived cell type, the EBs were treated with *trans*-retinoic acid which enhances neural differentiation (Levine and Flynn, 1986) and then plated on gelatin-coated dishes. One week after plating, cells with neuron-like morphology were found in the culture. Figure 4b shows neurite outgrowths in the Cr-1 (−/−) cell culture 2 weeks after plating. The axonal-like outgrowths expressed neurofilament transcripts because RT–PCR assays were positive (Fig. 5, lower panel) in differentiated cell cultures irrespective of Cr-1 expression.

To determine if inactivation of Cr-1 affects the differentiation of endoderm, the expression of α-fetoprotein (AFP), a marker for visceral endoderm cells, and laminin, a marker for parietal endoderm cells among other tissues, was not significantly different in mutant EBs at Day 8 from those in wild-type cells as determined by Western blotting analysis (Fig. 3a). Thus, Cr-1 is not required for differentiation of endoderm.

DISCUSSION

Functional and biochemical differentiation is readily studied using ES cells because of their ability to differentiate into a range of cell types in culture and into teratocarcinomas in adult hosts. The *in vitro* model of cardiogenesis using ES cells has been well characterized and the temporal stages in cardiomyocyte differentiation and function have been mapped, although spatial signals and morphogenesis

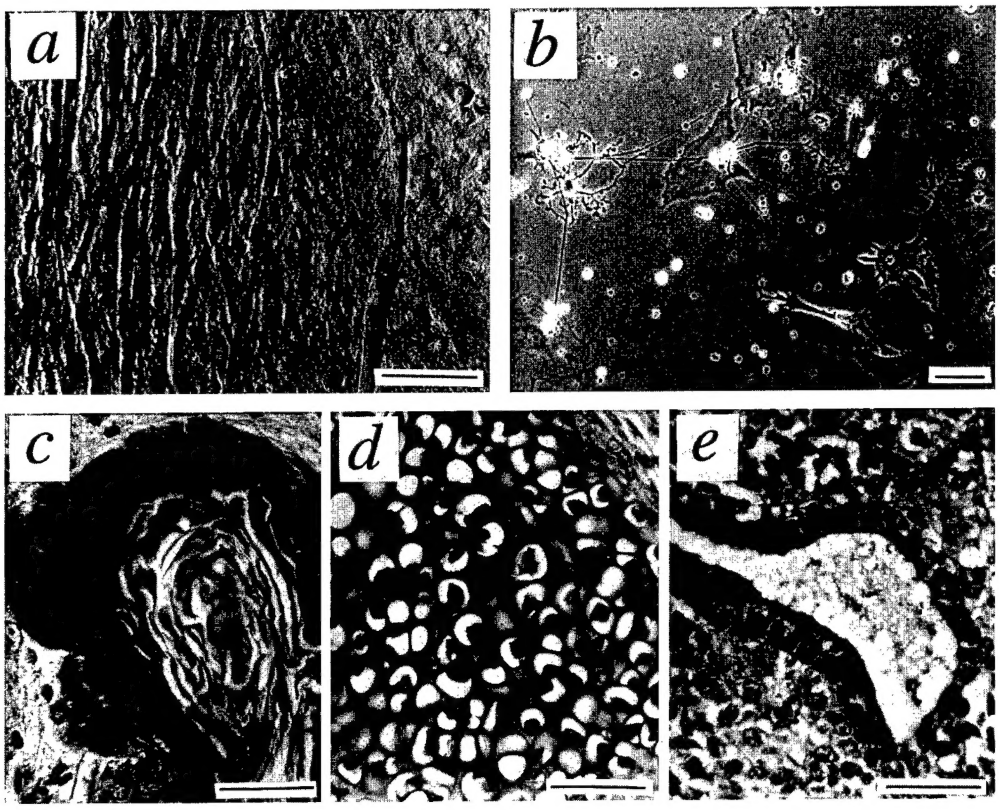

FIG. 4. *In vitro* and *in vivo* differentiation of Cr-1 (−/−) cells. (a) Differentiation of Cr-1 (−/−) cells into skeletal muscle. EBs derived from the Cr-1 (−/−) cell line DE39 were formed by hanging drops and transferred to gelatin-coated dishes on Day 5. Strongly contracting myotubes around the attached EBs were observed after culture for an additional 2 weeks. (b) Differentiation of Cr-1 (−/−) cells into neurons. The EBs were also treated with 5×10^{-7} M retinoic acid on Days 5–8 and transferred to gelatin-coated dishes after partial dissociation with trypsin–EDTA. Neurons were formed after plating. (c, d, e) Stained sections (hematoxylin and eosin) of teratocarcinomas derived from Cr-1 (−/−) ES cells. The tumors derived from Cr-1 (−/−) DE39 cells were fixed, embedded, and sectioned at 5 μm. Sections stained with hematoxylin and eosin contained readily recognizable dermis and squamous epidermis (c), cartilage (d), and epithelioid organs (e). Bar, 100 μm.

are lacking in this model (Fassler *et al.*, 1996; Maltsev *et al.*, 1993; Metzger *et al.*, 1996; Miller-Hance *et al.*, 1993; Wobus *et al.*, 1995).

Based on our results of *in vitro* and *in vivo* differentiation of ES cells, we concluded that disruption of Cr-1 results in a specific defect in cardiac differentiation but there is no detectable defect in differentiation of other mesodermal, endodermal and ectodermal cell types. These results emphasize a potentially important function for Cr-1 in cardiomyocyte differentiation at an early stage of cardiac development, which is consistent with predictions based on the expression pattern of Cr-1 during mouse embryogenesis.

Several transcription factor genes expressed in precardiac mesoderm, such as Nkx2.5 (Biben and Harvey, 1997; Harvey, 1996), Mef2C (Lyons *et al.*, 1995); Edmondson *et al.*, 1994) (Lin *et al.*, 1997), GATA4 (Heikinheimo *et al.*, 1994; Kuo *et al.*, 1997; Molkentin *et al.*, 1997), eHAND (Cserjesi *et al.*, 1995), and dHAND (Srivastava *et al.*, 1997) were predicted to be responsible for the commitment of mesodermal cells to the cardiac lineage (reviewed in Baker and Lyons, 1996). However, inactivation of these genes in mice did not affect differentiation of cardiomyocytes, but affected later events in cardiogenesis (Lyons, 1996; Olson and Srivastava, 1996). All of the transcription factors that we assayed that were shown to be involved in cardiac development were transcribed in Cripto null ES cell cultures. Therefore Cripto acts independently of the expression of these genes. Other than bone morphogenetic protein (BMP4) in chicks (Schultheiss *et al.*, 1997) and mouse (Winnier *et al.*, 1995) and hepatocyte growth factor (HGF) in mouse (Rappolee *et al.*, 1996) very little is known about how growth factors affect early cardiogenesis.

The use of ES cells to model differentiation *in vitro* allows the identification of induced gene activity in a temporal fashion but, except for a few good examples, such as neuronal differentiation (Levine and Flynn, 1986), it is an inadequate model for defining the events in a single pathway leading to defined cell types. This is because, without ma-

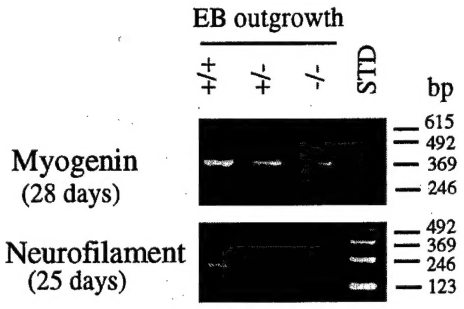

FIG. 5. RT–PCR analysis of myogenin and neurofilament expression in EB outgrowths. Cr-1 (+/+), Cr-1 (+/−), and Cr-1 (−/−) cell aggregates (EBs) were induced to differentiate into the structures shown in Fig. 4a, skeletal muscle, or Fig. 4b, neurons (see Materials and Methods). The cDNA samples from differentiated cultures were processed by PCR using primers specific for myogenin or neurofilament. The predicted DNA bands were produced in all cultures irrespective of Cr-1 expression.

nipulation, multiple differentiation pathways occur simultaneously. Fortunately for this analysis, cardiomyocyte differentiation is one of the earliest events and these cells start to beat on the seventh day of EB differentiation, reaching an incidence of >50% of total EBs. But even single EBs contain mixtures of several cell types and these differ between EBs and between cultures. Our finding that transcription factors thought to be specific to cardiac lineage are expressed in this mixture of cell types does not allow assignment of activities that are downstream or upstream of Cr-1 in cardiomyogenesis because independent pathways are mixed together. For instance, in our cultures there was always AFP-producing visceral endoderm, and these cells also express GATA-4 (Heikinheimo *et al.*, 1994). Primitive endoderm and thyroid primordium as well as precardiomyocytes express Nkx2.5 (Lints *et al.*, 1993). Mef2c is not specific to cardiac muscle since a low amount is also expressed in embryonic skeletal muscle and in neural crest cells (Edmondson *et al.*, 1994). Therefore, we found that several "cardiac-specifying" transcription factors are expressed in differentiating EBs that are unable to produce cardiac muscle. Indeed, transcripts for cardiac α-actin were detected in undifferentiated ES cells. This may have been due to leaky regulation of this gene and/or due to the presence of a few remaining feeder cells. For example, NIH 3T3 cells (mouse embryo-derived fibroblasts) express high levels of Nkx2.5 (Lints *et al.*, 1993). In our studies, feeder cells were removed by two rounds of differential plating. Fibroblast feeder cells in FBS-containing medium attach rapidly to plastic in contrast to ES cells, which are then collected from the supernatant medium. It is therefore unlikely that feeder cell contamination accounts for the presence of high levels of transcripts for cardiac α-actin.

The activation of muscle-specific genes, such as the muscle myosins and actins, is under the control of a large number of transcription factors, usually including MEF2 family members and serum response factor (SRF), and it is thought that specific combinations of factors modulate the type and activities of the developing muscle cells (Firulli and Olson, 1997). We describe here for the first time that inactivation of the growth factor gene, Cr-1, results specifically in defective early differentiation of cardiomyocytes due to the absence of contractile muscle proteins. This phenotype was specific to cardiogenesis because no other major tissue type was absent. The abrogation of the Cripto-1 gene was the cause of the phenotype because the reexpression of exogenous Cripto-1 in the "knockout" cells reversed the mutant phenotype. We speculate that Cripto-1 may be a master switch gene that activates the myosin genes in the cardiomyocyte via activation of the Ras/MAPK pathway (Kannan *et al.*, 1997) that could lead to activation of the SRF gene (Donoviel *et al.*, 1996) and other type-specific combinatorial transcription factor genes.

The unique specificity of Cripto-1 for cardiac cell differentiation is unusual because most factors activate both cardiac and skeletal muscle lineages. Possibly the EB provides the correct juxtaposition of primitive endoderm overlaying precardiac mesoderm, a combination shown to be heart-inductive in *Xenopus* (Nascone and Mercola, 1995). The result is that Cr-1 is produced and this, in turn, induces unknown target genes which in combination lead to cardiomyogenesis.

To devise strategies for cardiac muscle cell transplantation in heart disease treatments (Field, 1993) it will be helpful to define how specific growth factors such as Cr-1 promote cardiac lineages. Thus, these Cr-1 (−/−) and other targeted ES cell lines will provide a good system for studying specification and differentiation of the cardiac lineage and may provide information for therapy programs aimed toward regeneration of myocardium.

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